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DOI:

[10.1093/cid/ciz047](https://doi.org/10.1093/cid/ciz047)

*Document Version*

Peer reviewed version

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*Citation for published version (APA):*

Mehra, V., Rhone, E., Widya, S., Zuckerman, M., Potter, V., Raj, K., Kulasekararaj, A. G., McLornan, D. P., de Lavallade, H., Benson-Quarm, N., Lim, C., Ware, S., Sudhanva, M., Malik, O., Nicholas, R., Muraro, P. A., Marsh, J., Mufti, G. J., Silber, E., ... Kazmi, M. (2019). Epstein-Barr Virus and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis. *Clinical Infectious Diseases*, 69(10), 1757-1763. <https://doi.org/10.1093/cid/ciz047>

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# Clinical Infectious Diseases

## EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis --Manuscript Draft--

<b>Manuscript Number:</b>	CID-92283R2
<b>Full Title:</b>	EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis
<b>Short Title:</b>	EBV complications in Auto-HSCT for MS
<b>Article Type:</b>	Major Article
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<b>Manuscript Region of Origin:</b>	UNITED KINGDOM
<b>Abstract:</b>	<p><b>Introduction</b> Autologous haematopoietic stem cell transplantation (AHSCT) with anti-thymocyte globulin (ATG) conditioning as treatment of active multiple sclerosis (MS) is rapidly increasing across Europe (EBMT registry data 2017). Clinically significant Epstein Barr virus reactivation (EBV-R) following AHSCT with ATG for severe autoimmune conditions is an under-recognised complication relative to T-cell deplete transplants performed for haematological diseases. This retrospective study reports EBV-R associated significant clinical sequelae in MS patients undergoing AHSCT with rabbit ATG.</p> <p><b>Methods</b> Retrospective data was analysed for 36 consecutive MS-AHSCT patients at Kings College Hospital, London. All patients routinely underwent weekly EBV DNA PCR monitoring and serum electrophoresis for monoclonal gammopathy (MG or M-protein). EBV-R with rising EBV viral load, M-protein and associated clinical sequelae were captured from clinical records.</p> <p><b>Results</b> All patients had evidence of rising EBV DNA-emia, including 7 who were lost to long term follow-up, with a number of them developing high EBV viral load &amp; associated lymphoproliferative disorder (LPD). Nearly 72% (n-18/29) developed de-novo MG, some with significant neurological consequences with high M-protein and EBV-R. Six patients required anti-CD20 therapy (rituximab) with complete resolution of EBV related symptoms. Receiver operating characteristics (ROC) estimated a peak EBV viraemia of &gt;500,000 DNA copies/ml correlated with high sensitivity (85.5%) &amp; specificity (82.5%) (AUC-0.87; p-0.004) in predicting EBV-R related significant clinical events.</p> <p><b>Conclusion</b> Symptomatic EBV reactivation increases risk of neurological sequelae and LPD in MS-AHSCT. We recommend regular monitoring for EBV and serum electrophoresis for MG be mandated in MS patients in the first 3 months post AHSCT.</p>
<b>Response to Reviewers:</b>	<p>To Dr Barbara D Alexander M.D. Associate Editor Clinical Infectious Diseases</p> <p>Dated: 30th Dec 2018</p> <p>Dear Dr Alexander</p> <p>Subject: Response to Reviewers</p> <p>Manuscript Title: EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.</p> <p>We would like to thank the journal for provisionally accepting our work. Considering the reviewer's comments, we have made revisions to the manuscript with responses outlined for each of the queries raised by the reviewer, as below:</p> <p>The Authors simply must have the manuscript edited for English grammar as many of the mistakes change the meaning of the sentence. Some (but not all) of the issues are as follows: Response: Please accept our apologies for the grammatical errors in the manuscript. We have reviewed and edited these errors where appropriate including the ones highlighted below.</p> <p>Line 138 and line 357. deleted " be mandated". Cohort is too small to warrant "mandate"..but your data can lead to recommendation.. Response: We have edited and replaced the word 'mandate' from the phrase.</p> <p>Line 212-215. Please include the conversion factor for your assay to IU/ml in the methods section i.e 10 EBV DNA copies/ml=10 IU/ml</p>

Response:  
This has been rephrased within methods section; line 202-203

line 282: HAS versus IS?...I think "is"  
Response:  
Correction made to "is"

Line 298 and 300- not sure systemic sclerosis needs to be capitalized. But if so, needs to be so throughout manuscript  
Response:  
We have edited and removed un-necessary capitalisation for similar errors across the manuscript.

Lines 309-312. This sentence is not understandable based on current punctuation. Please address. ????This is further corroborated by the fact that similar LPD risk has not been observed in other ADs managed with ATG in our center. For example, among patients with Crohn' disease treated with ATG-AHSCT and those with severe aplastic anemia treated with ATG/cyclosporin, only 52% (x/x) developed EBV-R (unpublished data) and none had LPD, suggesting that the problem may not be ATG specific.  
Response:  
Thank you for the suggestion. We have rephrased this to reflect our experience with other Autoimmune diseases (lines 310-315).

Line 319 delete the words "may still have"  
Response:  
correction made.

Line 330 seems to "be"?  
Response:  
correction made.

Line 340 "copies"/ml  
Response:  
correction made.

Thank you again for your review of the revised manuscript. We hope these revisions are satisfactory and will allow formal acceptance for publication.

Yours sincerely

On behalf of all co-authors:

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To  
Professor Robert Schooley, M.D.  
The Editor-in-Chief  
Clinical Infectious Diseases

Dated: 20<sup>th</sup> Dec 2018

**Dear Professor Schooley (Editor-in-Chief) and Dr Alexander (Associate Editor)**

We are pleased to submit our revised article entitled; **"EBV & Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis"** for consideration of publication in your internationally reputed journal, Clinical Infectious Diseases.

Just to summarise again: Autologous Stem Cell Transplants (AHSCT) with anti-thymocyte globulin ATG) based conditioning is a novel approach to treatment of active multiple sclerosis (MS) and recent data from MIST study collaborators (Burt et al; Clinical Trial Registry: NCT00273364) have shown some exciting preliminary results showing superiority of AHSCT over established disease modifying therapies, confirming results from other UK and international studies in this field. However, as the evidence builds, safety aspects of these procedures needs to be seriously considered.

This study reports rates of Epstein Barr virus (EBV) reactivation and associated clinical sequelae with monoclonal gammopathy (M-protein), in cohort of Multiple Sclerosis patients who underwent ATG conditioned immunosuppressive AHSCT in a single centre. We report a significantly higher proportion of MS patients had detectable EBV DNA post-AHSCT; were more likely to develop clinically significant EBV viraemia of >500,000 DNA copies/ml and develop de-novo M-protein of clinical significance with clinical events ranging from probable lymphoproliferative disorders and disabling neurological complications, unrelated to MS. This report of significant clinical complications related to EBV and M-protein, possibly reflect underlying altered immunopathological state of MS disease and its interactions with reactivation of EBV virus, which if monitored and treated pre-emptively may reduce associated morbidity and improve outcomes.

To help readers, we have also described two interesting clinical vignettes as a supplementary to this report, highlighting significant risk of neurological events following development of M-protein, triggered following EBV reactivations in MS patients.

We can confirm that this manuscript has not been published and is not under consideration for publication elsewhere. All authors have seen, approved and contributed to this work. We have no conflicts of interest to disclose. We believe that this report fits well within the scope of your journal, highlighting important clinical message about EBV complications in ATG conditioned AHSCT for MS and will appeal to journal's readers interested in infectious complications related to immunosuppressive therapies including AHSCTs for autoimmune conditions, with a potential to change clinical practice in this area. We have provided point to point responses to the reviewer's comments.

Thank you for your consideration of this revised manuscript and looking forward to your acceptance.

Yours Sincerely

On behalf of all co-authors:

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# EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.

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61

62 Running Title: EBV complications in Auto-HSCT for MS  
63

64 Summary: EBV reactivation is common post-transplant with ATG for multiple sclerosis  
65 (MS), with significant lymphoproliferative & neurological sequelae associated with rising M-  
66 protein. Serial monitoring of EBV & M-protein is recommended post-transplant, as is pre-  
67-emptive anti-CD20 therapy with EBV DNA >500,000 copies/ml.

## 81 **Abstract**

### 82 **Introduction**

83 Autologous haematopoietic stem cell transplantation (AHSCT) with anti-thymocyte globulin  
84 (ATG) conditioning as treatment of active multiple sclerosis (MS) is rapidly increasing  
85 across Europe (EBMT registry data 2017). Clinically significant Epstein Barr virus  
86 reactivation (EBV-R) following AHSCT with ATG for severe autoimmune conditions is an  
87 under-recognised complication relative to T-cell deplete transplants performed for  
88 haematological diseases. This retrospective study reports EBV-R associated significant  
89 clinical sequelae in MS patients undergoing AHSCT with rabbit ATG.

### 90 **Methods**

91 Retrospective data was analysed for 36 consecutive MS-AHSCT patients at Kings College  
92 Hospital, London. All patients routinely underwent weekly EBV DNA PCR monitoring and  
93 serum electrophoresis for monoclonal gammopathy (MG or M-protein). EBV-R with rising  
94 EBV viral load, M-protein and associated clinical sequelae were captured from clinical  
95 records.

### 96 **Results**

97 All patients had evidence of rising EBV DNA-emia, including 7 who were lost to long term  
98 follow-up, with a number of them developing high EBV viral load & associated  
99 lymphoproliferative disorder (LPD). Nearly 72% (n-18/29) developed de-novo MG, some  
100 with significant neurological consequences with high M-protein and EBV-R. Six patients  
101 required anti-CD20 therapy (rituximab) with complete resolution of EBV related symptoms.  
102 Receiver operating characteristics (ROC) estimated a peak EBV viraemia of >500,000 DNA



103 copies/ml correlated with high sensitivity (85.5%) & specificity (82.5%) (AUC-0.87; p-0.004)  
104 in predicting EBV-R related significant clinical events.

## 105 **Conclusion**

106 Symptomatic EBV reactivation increases risk of neurological sequelae and LPD in MS-  
107 AHSCT. We recommend regular monitoring for EBV and serum electrophoresis for MG in  
108 MS patients in the first 3 months post AHSCT

## 109 **Key Words:**

110 **Multiple Sclerosis; Autologous Hematopoietic Stem Cell Transplantation, Epstein-**  
111 **Barr Virus Infection; Monoclonal Gammopathy; Post-transplant Lymphoproliferative**  
112 **Disorder**

113

114

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## 120 INTRODUCTION:

121 Multiple sclerosis (MS) is a chronic autoimmune, inflammatory, demyelinating disease of  
122 the central nervous system[1][2], with a relapsing-remitting (RRMS) presentation in the  
123 majority of patients at diagnosis. Recovery from relapses may be complete or partial[3][4].  
124 After a variable period of time, people with RRMS may develop a more progressive  
125 disability accumulation with or without superimposed relapses; termed secondary  
126 progressive multiple sclerosis (SPMS). A minority experience progressive disability from the  
127 onset of disease, termed primary progressive multiple sclerosis (PPMS)[4]. A number of  
128 immunomodulatory disease modifying therapies (DMTs) are currently licensed for treatment  
129 of RRMS with an aim of reducing number of relapses and accrual of disability, although with  
130 variable efficacy[5]. Since 1996, Autologous Hematopoietic Stem Cell Transplantation  
131 (AHSCT) has been a novel approach for MS management, using immunoablation followed  
132 by immunomodulation mechanisms, with evidence of significant suppression of  
133 inflammatory activity and qualitative changes in the reconstituted immune system (immune  
134 reset theory)[6–8]. AHSCT appears most effective for MS patients with evidence of  
135 inflammatory activity on MRI, younger age, a shorter disease duration, low to moderate  
136 disability levels (Expanded Disability Status Scale [EDSS] <6 or up to 6.5 if recent  
137 progression) and failure of at least 1 highly active DMT (natalizumab or alemtuzumab) with  
138 no significant comorbidities[9–11]. Recently reported preliminary results of randomised  
139 MIST study[12] found AHSCT to be superior to standard disease modifying therapy (DMT)

140 for RRMS with respect to both treatment failure and disability progression.

141

142 However, risk of subsequent rise in opportunistic infections following such  
143 immunosuppressive therapies remain a potential concern[13]. MS patients undergoing  
144 AHSCT have often been exposed to a number of immunomodulating DMTs; the addition of  
145 immunosuppressive rabbit anti-thymocyte globulin (rATG) to their conditioning regimen may  
146 confer a higher risk of viral reactivation in these patients. The number of AHSCTs  
147 performed for MS is rising significantly in Europe[14] and as more centres perform AHSCT  
148 for this indication, it is increasingly important to recognise the unique problems faced by  
149 these patients post AHSCT. This retrospective study reports for the first time, EBV-R  
150 associated neurological sequelae and lymphoproliferative disorder (LPD) in MS patients  
151 undergoing rATG conditioned AHSCT in our centre.

152

## 153 **METHODS**

### 154 **Patients and procedures**

155 Data was collected retrospectively on 36 consecutive MS patients undergoing AHSCT  
156 between February 2012 and February 2017 at Kings College Hospital, London. Peripheral  
157 blood stem cells were collected following standard mobilisation strategy consisting of  
158 cyclophosphamide 4g/m<sup>2</sup> over 2 days and granulocyte colony-stimulating factor for 7 days.  
159 Thirty-five (97%) patients were conditioned using standard protocol of cyclophosphamide

160 (50mg/kg for 4 days) and rATG (2.5mg/kg/day for 3 days) for in-vivo lymphodepletion  
161 followed by stem cell infusion. One patient was conditioned with  
162 carmustine/etoposide/cytarabine/melphalan regimen along with an equivalent dose of rATG  
163 (BEAM-ATG) prior to stem cell infusion. The median CD34 stem cell dose returned was  
164  $7.17 \times 10^6/\text{kg}$  (range  $4.0\text{-}17.1 \times 10^6/\text{kg}$ ).

165

166 Prior exposure to EBV was assessed by serological evidence (EBV viral capsid antigen;  
167 VCA IgG). EBV DNA load monitoring was performed on whole blood samples by  
168 standardised quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-  
169 Gene™ (Qiagen) assay of EBV BZLF1 DNA. This assay was adapted from our published  
170 assay using LightCycler (Roche)[15] and since been validated against the recently  
171 published WHO standard, with our lab's EBV DNA quantification of 10 copies/ml considered  
172 equivalent to 10 IU/ml DNA reported with the WHO reference method[16]. EBV-R was  
173 defined as rising EBV DNA load of  $>10$  copies/millilitre (ml) detected on two consecutive  
174 tests based on our assay sensitivity. Symptomatic EBV-R was captured by peak blood EBV  
175 DNA load, presence of B symptoms (defined by presence of either unexplained weight loss,  
176 recurrent fever, night sweats); which was in-turn defined by clinical, radiological and/or  
177 histological evidence based on recent ECIL-6 guidelines[17]. In addition, significant 'clinical  
178 events' were also defined as new & persistent organ dysfunction (e.g. neurological events)  
179 temporally associated with rising EBV viraemia in MS patients. Serum protein  
180 electrophoresis was routinely tested around 3 months post HSCT as part of our institutional  
181 practice, with immunoglobulin subclasses identified by immunofixation electrophoresis.  
182 Patient outcomes were assessed at last follow up as of April 2017.

183

## 184     **Statistics**

185     The database of transplants and outcomes was built in Microsoft Excel 2016 and statistical  
186     analyses were performed using IBM SPSS Statistics version 24.0. Patient characteristics  
187     are presented as medians (with inter-quartile ranges; IQR) for data with non-normal  
188     distribution. Comparisons of baseline characteristics used Mann-Whitney U test, Fisher's  
189     exact test, or Chi-squared test for trend as appropriate. Receiver Operating characteristics  
190     (ROC) curve was obtained correlating LPD and clonal gammopathy associated clinical  
191     events with rising EBV viraemia (copies/ml).

192

## 193     **RESULTS**

194     Baseline characteristics are presented in **Table 1**. Most MS patients (88.9%) had RRMS  
195     phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients  
196     had prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients  
197     received both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-  
198     treatment, indicating prior EBV exposure and had detectable EBV DNA post-AHSCT.  
199     Seven MS patients were lost to long-term follow up for EBV monitoring. The median time to  
200     first EBV DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels  
201     peaked at a median of 32 days post-transplant (IQR 31-53 days). All MS patients had  
202     normal baseline lymphocyte counts (median) pre-HSCT with a median time of 46 days  
203     (range 14-404 days) to lymphocyte recovery (defined by total lymphocyte count  
204      $>1.0 \times 10^6/\text{ml}$ ) following AHSCT (**See Figure 1**). A high proportion (86%; n=25/29) of the MS  
205     patients in active follow-up recovered lymphocyte counts around D56 with a median

206 lymphocyte count of  $1.56 (10^6 \text{ cells/ml})$ ; Four patients remained lymphopenic at last follow  
207 up.

208

209 All patients were stratified into following 3 groups according to peak rise/burden of EBV  
210 DNA-aemia (copies/ml):  $<100,000 (<100k) \text{ copies/ml}$ ,  $100,001-500,00 (100k-500k)$   
211  $\text{copies/ml}$  and  $>500,000 (>500k) \text{ copies/ml}$  to identify any specific thresholds for clinically  
212 significant events related to rising EBV-R (Table 1). The majority of patients (76%) with  
213 rising EBV viral load  $>100k \text{ copies/ml}$  were routinely screened by computed tomographic  
214 (CT) scans to assess for evidence of LPD, in line with our institutional policy. One third  
215 (34.5%) of patients developed peak EBV viraemia of  $>500k \text{ copies/ml}$ . Eight patients  
216 (27.6%) developed symptomatic EBV-R; defined as persistent fever, lymphadenopathy  
217 and/or B symptoms. Of these 8 patients, only 1 (12.5%) had a peak EBV viraemia  
218  $<100k \text{ DNA copies/ml}$  with the remaining 7 (87.5%) patients having a peak EBV viraemia of  
219  $>500k \text{ copies/ml}$ . Three patients with rising EBV viraemia  $>500k \text{ copies/ml}$  had findings  
220 consistent with probable LPD on CT imaging; however, none had definitive histological  
221 diagnosis. Three MS patients had worsening neurological symptoms concurrent with rising  
222 EBV viraemia  $>500k \text{ copies/ml}$  and clonal gammopathy, as described below.

223

224 Interestingly, we also observed frequent de novo monoclonal gammopathy (MG or M-  
225 protein) in 18 MS patients (62.4%) following rising EBV viraemia; the majority (n-16) of

226 whom developed IgG subtype and the remaining 2 developed IgA and IgM M-protein.  
227 Concerningly two of these patients developed clinically significant M-Protein burden; one  
228 patient with IgG Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological  
229 symptoms mimicking MS relapse, requiring plasma exchange. Another patient developed  
230 significant lumbosacral radiculopathy with rising EBV-R and high IgM lambda M-protein  
231 (IgM 48.5g/L) (**see supplementary case vignettes**). **Figure 2** highlights the association of  
232 neurological symptom onset following rising EBV viraemia (log copies), falling lymphocyte  
233 counts ( $\times 10^6/\text{ml}$ ) with significant rise in M-protein (gm/lt) levels post AHSCT. A third patient  
234 developed painful lower limb paraesthesia following rising EBV viraemia  $>500\text{k}$  copies/ml,  
235 although did not have any M-protein detected. Their symptoms persisted at last follow up  
236 despite no evidence of MS related new disease activity.

237

238 Six patients were treated with anti-CD20 antibody, rituximab (375mg/m<sup>2</sup> weekly up to 4  
239 weeks), due to clinical severity of EBV reactivations and leading to reduction in EBV viral  
240 load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis  
241 (**Figure 3**) confirmed EBV viraemia of  $>500\text{k}$  copies/ml correlated with high sensitivity  
242 (85.5%) and specificity (82.5%) (AUC-0.87; 95%CI-0.73-1.0; p-0.004) in predicting  
243 significant EBV related clinical events (evidence of LPD and/or neurological symptoms) that  
244 may require treatment with rituximab. The sensitivity dropped significantly on lower  
245 estimates for events below 500k copies/ml.

246

247 The median time to resolution of EBV viraemia post-rituximab was 21 days (IQR 19-124  
248 days) in 5 patients with >500k copies/ml (one patient was treated for late onset persistent  
249 symptomatic clonal gammopathy, despite fall in EBV levels). No significant adverse events  
250 were noted in the treated group. Nine patients had a persistent low level EBV viraemia  
251 detectable at last follow up. All patients who underwent AHSCT were alive as of April 2017.

252

## 253 **DISCUSSION:**

254 MS as an autoimmune disorder (AD) is theorised to have generally similar underlying  
255 pathophysiological immune dysregulation mechanisms[18–22] relative to other chronic  
256 autoimmune conditions. Epstein-Barr virus (EBV) is increasingly implicated in the  
257 pathogenesis of MS by virtue of epidemiological and serological evidence, impaired CD8+  
258 T cell immune responses to EBV and possible underlying genetic susceptibility for  
259 autoimmunity (with EBV encoded protein interactions), as recently described by Harley et al  
260 and others[1,23–25].

261

262 Generally, EBV-R related LPD has been reported in both allogeneic (allo-HSCT) and solid  
263 organ transplants treated with immunosuppressive therapy, often with a significant impact  
264 on organ function and overall survival[26–30]. It is observed that reduced intensity allo-  
265 HSCT for malignant haematological conditions using alemtuzumab have a relatively lower



266 overall risk of LPD compared to ATG based treatments, possibly mediated by more  
267 effective pan-B & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell  
268 repertoire with delayed EBV specific CD8+ T cell recovery[31]. Clinically significant  
269 endogenous viral infections including EBV following ATG conditioned AHSCT for severe  
270 ADs such as Crohn's disease and systemic sclerosis is increasingly recognised, but the  
271 development of lymphoproliferative disorders (LPD) remains rare in these ADs[13,32,33].  
272 Nash et al[32] concerningly reported 2 deaths (1 MS and 1 systemic sclerosis patient) from  
273 EBV related LPD in 56 ATG conditioned AHSCT for autoimmune diseases. Additionally,  
274 EBV associated haemophagocytosis in ATG-AHSCT for ADs have also been reported[34],  
275 with one resulting in death of the patient[35].

276

277 Our report of suspected EBV related LPDs (~10%) in MS-AHSCT group is relatively higher  
278 than published reports with allo-HSCTs (4.5-7%)[36,37] and our own centre's unpublished  
279 T-cell depleted allo-HSCT experience (6.5%); possibly a reflection of underlying  
280 immunopathological state of MS itself[38]. This is further corroborated by the fact that  
281 similar LPD risk has not been observed in other ADs, e.g. Crohn's disease, treated with  
282 ATG -AHSCT in this centre. Another example from our centre's experience of severe  
283 aplastic anaemia (n=40) treated with ATG/ciclosporin, only 52% (n=21/40) developed EBV-R  
284 (unpublished data) and none had LPD or required any treatment, suggesting that the  
285 problem may not be ATG specific.

286

287 Our study's observation of significant persistent neurological events (with no evidence of  
288 new MS disease activity) associated with clonal gammopathy suggest a potentially new  
289 clinical syndrome, described for the first time in ATG conditioned AHSCTs in MS and  
290 possibly induced by clonal B cell dysregulation following EBV-R. It could be hypothesised  
291 that any remaining EBV infected latent B cells, surviving despite high doses of  
292 cyclophosphamide (given with mobilisation and conditioning in MS ASHCT)[8] and  
293 compounded by depletion of CD8+ T cells by ATG, may serve as potential source for EBV  
294 escape while interacting abnormally within the host immune micro-environment[39] and  
295 leading to rise in M-protein, LPD and neuro-inflammatory insults in some of the MS patients  
296 post AHSCT. Reports of lower incidence of EBV-R and LPD in another commonly used  
297 protocol for MS-AHSCT using BEAM-ATG (Ricardo Saccardi, Carregi University Hospital,  
298 Florence; personal communication) may reflect the greater myeloablative effect of BEAM  
299 chemotherapy which could further deplete the residual B cell pool and thus lower potential  
300 for EBV proliferation. It is plausible that dose of rATG is critical, given we have not seen  
301 similar reports from other centres where less rATG doses were given for MS-AHSCT (range  
302 between 5.0-6.5 mg/Kg; personal communication) but there seems to be some variability in  
303 prospective serial EBV monitoring in these patients.

304

305 The clinical threshold for EBV viral load as a significant risk factor for post-transplant LPD is  
306 widely debated. This study reports peak EBV viral load >500k copies/ml post AHSCT is  
307 significantly associated with probable LPD and neurological events in MS patients with high  
308 sensitivity (85.5%) and specificity (82.5%) (p=0.004) (**Fig 3, ROC curve**). Our ROC curve  
309 estimates are potentially limited by the relatively small number of events analysed but this  
310 has consistently been useful in our MS-AHSCT experience for predicting clinical events  
311 with high EBV load. Our EBV PCR assay has been validated against the recently defined  
312 standard WHO reference method (i.e. 10 copies/ml=10 IU/ml EBV DNA) [16] and thus this  
313 EBV threshold for pre-emptive treatment with Rituximab, can potentially be applied in  
314 relevant clinical context in other centres using similar validated essays. Rituximab treatment  
315 delivered good overall response in our symptomatic patients, with resolution of EBV related  
316 clinical symptoms and no subsequent viral or bacterial infections at last follow up. The role  
317 of prophylactic or pre-transplant rituximab in MS-AHSCT is also a potential area of interest  
318 in reducing risk of EBV-LPD in stem cell transplants as observed by Burns et al[36] and  
319 future randomised studies are required to investigate its potential benefit.

320

321 Our study limitations include its retrospective nature and that no suspected LPD patients  
322 had histological confirmation, mainly related to patient refusal or technical difficulties. Seven  
323 MS patients were lost to follow up for EBV monitoring following discharge, which limits the

324 findings of this study. Additionally, our numbers were too small to identify any association of  
325 EBV related clinical events with previous DMT exposure in MS patients.

326

327 In conclusion, symptomatic EBV reactivation increases risk of neurological sequelae and  
328 LPD in MS-AHSCT. Regular monitoring for rising EBV viraemia, as recommended by  
329 Snowden et al [10] and Muraro et al [11], and serum electrophoresis for M-protein should  
330 be considered in the first 3 months post-AHSCT for MS. We recommend persistent high  
331 EBV viraemia > 500k DNA copies/ml as potential trigger for consideration of pre-emptive  
332 anti-CD20 therapy and potentially reduce associated morbidity.

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340 **Acknowledgements:** To our patients and their families and carers in supporting this study.

341 **Conflict of interest:** The authors declare no competing financial interests as below:

- 342 1. **Varun Mehra-** no competing financial interests
- 343 2. **Elijah Rhone-** no competing financial interests
- 344 3. **Stefani Widya-** no competing financial interests
- 345 4. **Mark Zuckerman-** no competing financial interests
- 346 5. **Victoria Potter-** no competing financial interests
- 347 6. **Kavita Raj-** no competing financial interests
- 348 7. **Austin Kulasekararaj-** no competing financial interests
- 349 8. **Donal McLornan-** no competing financial interests
- 350 9. **Hugues de Lavallade-** no competing financial interests
- 351 10. **Nana Benson-Quarm-** no competing financial interests
- 352 11. **Christina Lim-** no competing financial interests
- 353 12. **Sarah Ware-** no competing financial interests
- 354 13. **Malur Sudhanva-** no competing financial interests
- 355 14. **Omar Malik-** no competing financial interests
- 356 15. **Richard Nicholas-** no competing financial interests
- 357 16. **Paolo A Muraro-** no competing financial interests
- 358 17. **Judith Marsh-** no competing financial interests
- 359 18. **Ghulam J Mufti-** no competing financial interests
- 360 19. **Eli Silber-** no competing financial interests
- 361 20. **Antonio Pagliuca-** no competing financial interests
- 362 21. **Majid A. Kazmi-** no competing financial interests

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520 **Table 1: Baseline patient characteristics and EBV related clinical events according to peak**  
521 **EBV DNA-aemia burden.**

Baseline characteristics (n-36)		Patient Groups according to peak EBV DNA in copies/ml (n-29)	0 - 100,000	100,001 - 500,000	>500,000
Median age at time of AHSCT in years (range)	43.5 (36– 47)	No of patients (%)	16 (55.2)	3 (10.3)	10 (34.5)
Gender		M-Protein (n)	11	0	7
Male	19 (52.8%)				
Female	17 (47.2%)				
Disease Type (n; %)		Median EBV DNA log value at peak (IQR)			
Relapsing Remitting MS	22 (61.1%)		4.8 (3.5-4.8)	5.5 (N/A)	6.25 (6.1-6.9)
Secondary Progressive MS	10 (27.8%)				
Primary Progressive MS	4 (11.1%)				
Median number of previous DMT (range)	2 (0 – 6)	Median number of prior DMTs	2	3	2
Previous use of high efficacy DMT (n)		Symptomatic EBV (n)			
Natalizumab	22		1	0	7
Alemtuzumab	8				
Both	6				
Median EDSS (range)	6.0 (2.5 – 8.0)	LPD diagnosis (CT/Biopsy) (n)	0	0	3 by CT alone
Median follow up post AHSCT in days (range)	436 (188 – 785)	Neuro/autoimmune complications (n)	0	0	3
No. of patients with prior EBV exposure (n; %)	36 (100%)	Treated with Rituximab (n)	0	0	6
No. of patients with detectable EBV post AHSCT (n; %)	29 (80.5%)	Confirmed EBV resolution at last follow up (n)	7	2	7
No. of patients lost to long term follow up (n; %)	7 (19.5%)	Detectable EBV DNA at last follow up (n)	9	1	3
Median Time to EBV detection post AHSCT in days (IQR)	30 (23-46)	Median time for EBV resolution (IQR in days)	67 days (44-155)	47 days (N/A)	63 days (45 - 170)
Median Time to peak EBV DNA levels in days (IQR)	32 (31-53)	Median Time to peak EBV DNA levels in days (IQR)	40 days (25-85)	30 days (N/A)	39 days (32-43)

523

524 **Abbreviations:**

525 AHSCT- Autologous Haematopoietic Stem cell transplant; CT- computed tomography;  
526 DMT- Disease modifying therapy; EBV- Epstein Barr virus; EDSS- Kurtzke Expanded  
527 Disability Status Scale; IQR- Interquartile range; LPD- Lymphoproliferative disease; M-  
528 Protein: Monoclonal paraprotein or gammopathy; MS- Multiple Sclerosis

## Figure Legends

### Figure 1: Trend of Lymphocyte count recovery following ATG in MS patients.

**Legend:** This figure shows trends of lymphocyte count from baseline to recovery post AHSCT for MS patients. Majority of patients had normal baseline lymphocyte counts pre-HSCT and became lymphopenic post ATG with counts recovering towards d+28 and >85% of patients recovered counts by d+56, with some overshooting from their baseline, possibly reflective of EBV related lymphoproliferation in some of these patients.

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

### Figure 2: Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT.

**Legend:** This figure demonstrates trends of EBV copies (log), paraprotein levels (g/l) and Lymphocyte levels (counts  $\times 10^6/\text{ml}$ ) in two MS patients with significant neurological symptoms following EBV reactivation. Both patients had significant EBV viraemia ( $\log > 5.2$  or  $> 500,000$  copy number) and developed significant paraproteinaemia, which was only noted after persistent unexplained neurological symptoms. The trend reversed following anti-CD20 (rituximab) therapy, with limited recovery in neurological symptoms.

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

### Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.

**Legend:** ROC curve demonstrating significant correlation between high EBV levels and clinical events (LPD & neurological events) in MS-AHSCT patients, with highest sensitivity and specificity noted with peak EBV viraemia of  $> 500,000$  copies/ml ( $p = 0.0004$ ).

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

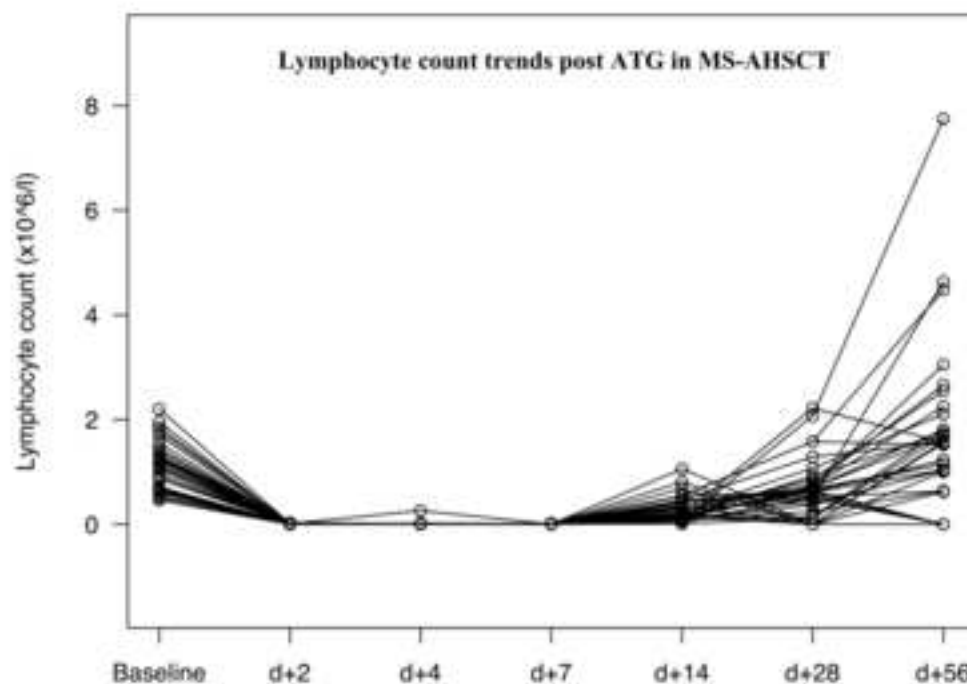
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**Abbreviations:**

AHSCT- Autologous Haematopoietic Stem cell transplant; CT- computed tomography; DMT- Disease modifying therapy; EBV- Epstein Barr virus; EDSS- Kurtzke Expanded Disability Status Scale; IQR- Interquartile range; LPD- Lymphoproliferative disease; M-Protein: Monoclonal paraprotein or gammopathy; MS- Multiple Sclerosis

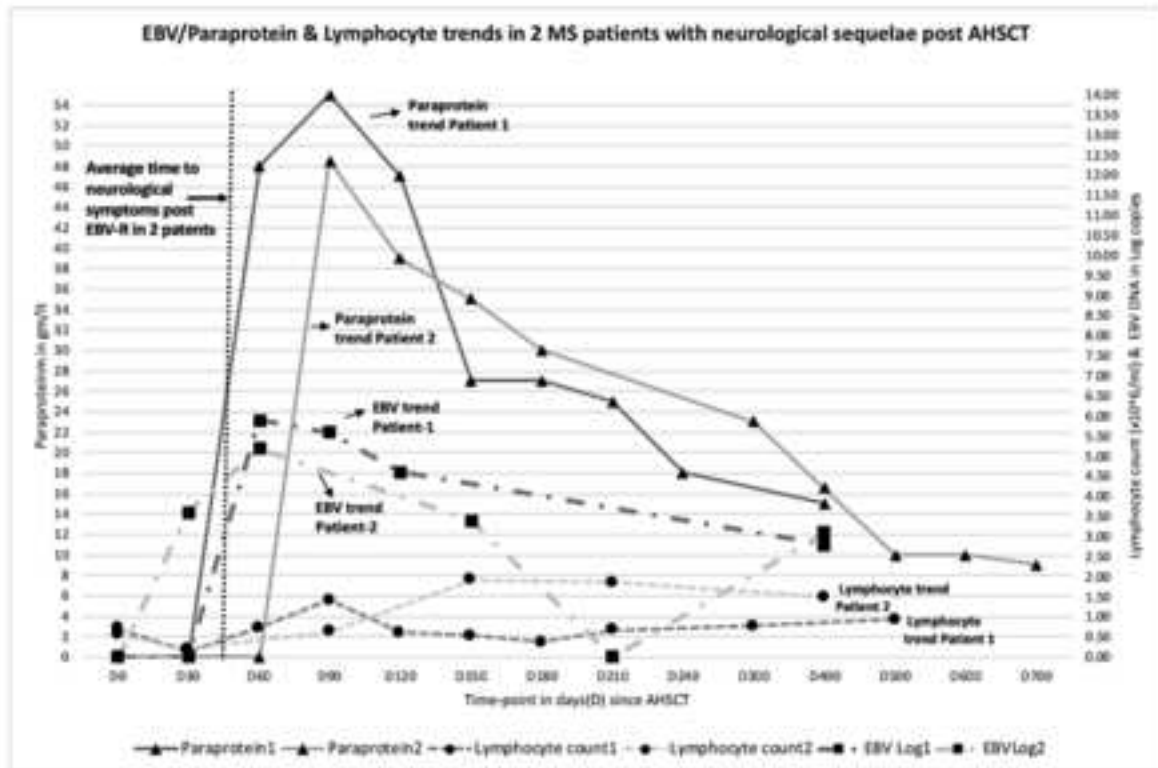
**Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in MS patients.**



**Abbreviations:**

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

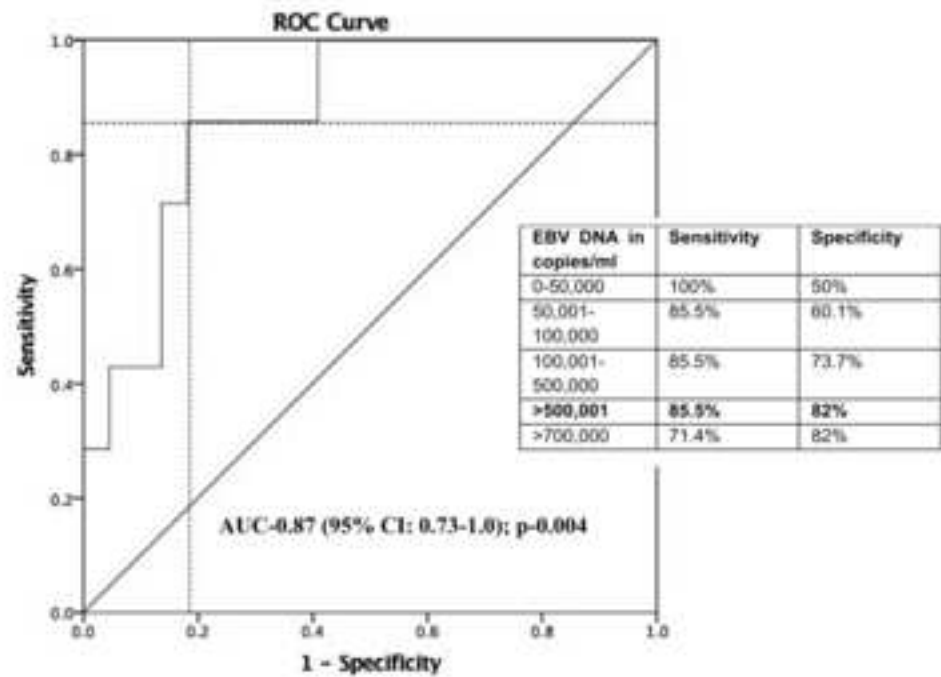
**Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT**



#### Abbreviations:

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

**Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.**



**Abbreviations:**

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

## **EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.**

*Running Title: EBV complications in Auto-HSCT for MS*

### **SUPPLEMENTARY:**

**Case vignettes of 2 MS patients describing EBV-related significant paraproteinaemia and neurological sequelae.**

#### **Patient 1**

43-year-old female with Relapsing Remitting Multiple Sclerosis (RRMS), previously treated with natalizumab and three courses of alemtuzumab but continued to have breakthrough disease. She had a relatively mild baseline disability with an Expanded Disability Severity Scale (EDSS) of 2.5. She had an uncomplicated inpatient stay for the Autologous Haematopoietic Stem cell transplant (AHSCT) procedure and was discharged on day 15 post-transplant. A blood test on day 26 demonstrated Epstein-Barr Virus (EBV) reactivation (155,845 copies/ml). A repeat test on day 34 showed an increase in copy number to 638,634 copies/ml. She was asymptomatic, and the plan was to monitor this closely. On day 37 post-transplant she developed a significant deterioration in strength in the right lower limb and on day 42 she developed pyrexia and was admitted to a local hospital. She was found to have CMV reactivation which was treated with IV ganciclovir as well as ongoing EBV reactivation and she remained an inpatient for 4 weeks. She did not receive rituximab at the local centre but on repeat testing at day 145 the copy number was vastly reduced at 2,355 copies/ml. A high IgM paraproteinaemia was first detected at day 92 post-transplant (48.58g/L). This had not been routinely monitored previously. This paraproteinaemia was initially felt to be asymptomatic and was monitored closely, slowly improving over time. A CT scan was performed which demonstrated a single 1.7cm right hilar lymph node requiring observation.



A bone marrow aspirate showed a small excess of plasma cells (5-9%) on aspirate with no other significant findings.

The EBV reactivation initially settled at 6 months post-transplant. At one-year post transplant she had a persistent IgM paraprotein (23g/L) and her right leg weakness had continued to progress with her EDSS now at 5.0. There was also a mild recurrence of EBV (DNA at 1,335 copies/ml). It was considered that as the onset of the right leg weakness had coincided with the high level of EBV reactivation and paraproteinaemia that these factors may have driven a peripheral neuropathy. She was treated with rituximab 375mg/m<sup>2</sup> weekly for 4 weeks at 19 months post-transplant following which EBV DNA again became undetectable and the paraprotein reduced to 9g/L. Despite this, there was no improvement in strength of the right leg. Nerve conduction studies subsequently confirmed an L5-S1 radiculopathy but without a generalised polyneuropathy neuropathy. She has had no new or active demyelinating lesions on MRI head and spine post-transplant that would account for these symptoms and the slowly progressive nature of the weakness does not suggest an MS relapse. The cause of the weakness is likely an atypical IgM paraprotein associated radiculo-neuropathy was strongly suspected.

## **Patient 2**

42-year-old male with Secondary Progressive Multiple Sclerosis (SPMS), previously treated with interferon and copaxone which were discontinued due to side effects and ongoing relapses, respectively. He was then treated with natalizumab for 2 years but continued to progress and was offered HSCT. He had a moderate level of baseline disability with an EDSS of 5.5 (walking at least 100m unaided). The transplant procedure was complicated by neutropenic sepsis which was treated successfully, and he was discharged on day 13 post-transplant with no new neurological symptoms. He was readmitted on day 17 post-transplant with pyrexia and rigors. Blood cultures grew *Stenotrophomonas maltophilia* and he was treated for line sepsis with appropriate antibiotics and fully recovered. An EBV viraemia of

58,324 copies/ml was detected for the first time on this admission. On day 22 he had developed new urinary urgency, diplopia and significant deterioration in mobility. This was felt to represent either a pseudorelapse driven by infection or a true relapse and an MRI was performed which demonstrated no new demyelinating lesions and no other significant pathology. A repeat EBV DNA assessment on day 28 demonstrated a significant rise in EBV viraemia to >10 million copies/ml (log change).

His neurological symptoms persisted and on day 34 he began spiking temperatures again; antibiotics were restarted but blood and urine cultures came back negative, but his EBV viraemia had risen to over 39 million copies/ml. He continued to experience intermittent pyrexia, which possibly was attributed to his EBV viraemia. No evidence of lymphadenopathy was noted during this period. Due to significant neurological decline, he was consequently commenced on rituximab 375mg/m<sup>2</sup> weekly for 4 weeks on day 38 post-transplant. Testing on day 51 demonstrated a reduction in EBV viraemia to DNA of 2.2 million copies/ml and on day 52, a significant IgG kappa paraproteinaemia (45.6 g/L) was identified. This had not been routinely monitored previously. It was considered that this degree of paraproteinemia and resulting hyperviscosity may have been a driver of his neurological symptoms. These values continued to improve over time with further doses of rituximab and the EBV viraemia was <100,000 copies/ml and the IgG kappa paraprotein down to 8.63 g/L by Day 87. However, due to persistence of these markers as well as his ongoing neurological symptoms, he was given a single plasma exchange on day 80 that was of minimal symptomatic benefit.

He had ongoing rehabilitation, including a short admission in a specialist neuro-rehabilitation ward. neurorehabilitation unit. At one year review he still required bilateral support to walk, putting his EDSS at 6.5. A repeat MRI at 12 months post-transplant was again stable with no new demyelinating lesions. This patient demonstrated significant deterioration in his condition post-transplant and although there may be an element of disease progression, we suspect this was in large part driven by EBV viraemia and associated paraproteinaemia/hyperviscosity.

The EBV viraemia was undetectable at the last follow up, although there was ongoing paraproteinaemia with an IgG kappa of 15 g/L.

# **EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.**

***Running Title:*** *EBV complications in Auto-HSCT for MS*

**Varun Mehra\*, Elijah Rhone\*, Stefani Widya, Mark Zuckerman, Victoria Potter, Kavita Raj, Austin Kulasekararaj, Donal McLornan, Hugues de Lavallade, Nana Benson-Quarm, Christina Lim, Sarah Ware, Malur Sudhanva, Omar Malik, Richard Nicholas, Paolo A Muraro, Judith Marsh, Ghulam J Mufti, Eli Silber, Antonio Pagliuca and Majid A. Kazmi**

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## ***Key Points:***

**EBV reactivation is common post-transplant with ATG for multiple sclerosis (MS), with significant lymphoproliferative & neurological sequelae associated with rising M-protein. Serial monitoring of EBV & M-protein is recommended post-transplant, as is pre-emptive anti-CD20 therapy with EBV DNA >500,000 copies/ml.**

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## ***Key Words:***

**Multiple Sclerosis; Autologous Hematopoietic Stem Cell Transplantation, Epstein-Barr Virus Infection; Monoclonal Gammopathy; Post-transplant Lymphoproliferative Disorder**

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78 **Conflict of interest:** The authors declare no competing financial interests as below:

- 79 1. **Varun Mehra-** no competing financial interests
- 80 2. **Elijah Rhone-** no competing financial interests
- 81 3. **Stefani Widya-** no competing financial interests
- 82 4. **Mark Zuckerman-** no competing financial interests
- 83 5. **Victoria Potter-** no competing financial interests
- 84 6. **Kavita Raj-** no competing financial interests
- 85 7. **Austin Kulasekararaj-** no competing financial interests
- 86 8. **Donal McLornan-** no competing financial interests
- 87 9. **Hugues de Lavallade-** no competing financial interests
- 88 10. **Nana Benson-Quarm-** no competing financial interests
- 89 11. **Christina Lim-** no competing financial interests
- 90 12. **Sarah Ware-** no competing financial interests
- 91 13. **Malur Sudhanva-** no competing financial interests
- 92 14. **Omar Malik-** no competing financial interests
- 93 15. **Richard Nicholas-** no competing financial interests
- 94 16. **Paolo A Muraro-** no competing financial interests
- 95 17. **Judith Marsh-** no competing financial interests
- 96 18. **Ghulam J Mufti-** no competing financial interests
- 97 19. **Eli Silber-** no competing financial interests
- 98 20. **Antonio Pagliuca-** no competing financial interests
- 99 21. **Majid A. Kazmi-** no competing financial interests

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## 111 **Abstract**

### 112 **Introduction**

113 Autologous haematopoietic stem cell transplantation (AHSCT) with anti-thymocyte globulin  
114 (ATG) conditioning as treatment of active multiple sclerosis (MS) is rapidly increasing across  
115 Europe (EBMT registry data 2017). Clinically significant Epstein Barr virus reactivation (EBV-  
116 R) following AHSCT with ATG for severe autoimmune conditions is an under-recognised  
117 complication relative to T-cell deplete transplants performed for haematological diseases.  
118 This retrospective study reports EBV-R associated significant clinical sequelae in MS patients  
119 undergoing AHSCT with rabbit ATG.

### 120 **Methods**

121 Retrospective data was analysed for 36 consecutive MS-AHSCT patients at Kings College  
122 Hospital, London. All patients routinely underwent weekly EBV DNA PCR monitoring and  
123 serum electrophoresis for monoclonal gammopathy (MG or M-protein). EBV-R with rising  
124 EBV viral load, M-protein and associated clinical sequelae were captured from clinical  
125 records.

### 126 **Results**

127 All patients had evidence of rising EBV DNA-emia, including 7 who were lost to long term  
128 follow-up, with a number of them developing high EBV viral load & associated  
129 lymphoproliferative disorder (LPD). Nearly 72% (n-18/29) developed de-novo MG, some with  
130 significant neurological consequences with high M-protein and EBV-R. Six patients required  
131 anti-CD20 therapy (rituximab) with complete resolution of EBV related symptoms. Receiver  
132 operating characteristics (ROC) estimated a peak EBV viraemia of >500,000 DNA copies/ml

133 correlated with high sensitivity (85.5%) & specificity (82.5%) (AUC-0.87; p-0.004) in  
134 predicting EBV-R related significant clinical events.

## 135 **Conclusion**

136 Symptomatic EBV reactivation increases risk of neurological sequelae and LPD in MS-  
137 AHSCT. We recommend regular monitoring for EBV and serum electrophoresis for MG ~~be~~  
138 ~~mandated~~ in MS patients in the first 3 months post AHSCT

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## 151 INTRODUCTION:

152 Multiple sclerosis (MS) is a chronic autoimmune, inflammatory, demyelinating disease of the  
153 central nervous system[1][2], with a relapsing-remitting (RRMS) presentation in the majority  
154 of patients at diagnosis. Recovery from relapses may be complete or partial[3][4]. After a  
155 variable period of time, people with RRMS may develop a more progressive disability  
156 accumulation with or without superimposed relapses; termed secondary progressive multiple  
157 sclerosis (SPMS). A minority experience progressive disability from the onset of disease,  
158 termed primary progressive multiple sclerosis (PPMS)[4]. A number of immunomodulatory  
159 disease modifying therapies (DMTs) are currently licensed for treatment of RRMS with [an](#)  
160 aim of reducing number of relapses and accrual of disability, although with variable  
161 efficacy[5]. Since 1996, Autologous Hematopoietic Stem Cell Transplantation (AHSCT) has  
162 been a novel approach for MS management, using immunoablation followed by  
163 immunomodulation mechanisms, with evidence of significant suppression of inflammatory  
164 activity and qualitative changes in the reconstituted immune system (immune reset theory)[6–  
165 8]. AHSCT appears most effective for MS patients with evidence of inflammatory activity on  
166 MRI, younger age, a shorter disease duration, low to moderate disability levels (Expanded  
167 Disability Status Scale [EDSS] <6 or up to 6.5 if recent progression) and failure of at least 1  
168 highly active DMT (natalizumab or alemtuzumab) with no significant comorbidities[9–11].  
169 Recently reported preliminary results of randomised MIST study[12] found AHSCT to be  
170 superior to standard disease modifying therapy (DMT) for RRMS with respect to both

171 treatment failure and disability progression.

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173 However, risk of subsequent rise in opportunistic infections following such  
174 immunosuppressive therapies remain a potential concern[13]. MS patients undergoing  
175 AHSCT have often been exposed to a number of immunomodulating DMTs; the addition of  
176 immunosuppressive rabbit anti-thymocyte globulin (rATG) to their conditioning regimen may  
177 confer a higher risk of viral reactivation in these patients. The number of AHSCTs performed  
178 for MS is rising significantly in Europe[14] and as more centres perform AHSCT for this  
179 indication, it is increasingly important to recognise the unique problems faced by these  
180 patients post AHSCT. This retrospective study reports for the first time, EBV-R associated  
181 neurological sequelae and lymphoproliferative disorder (LPD) in MS patients undergoing  
182 rATG conditioned AHSCT in our centre.

183

## 184 **METHODS**

### 185 **Patients and procedures**

186 Data was collected retrospectively on 36 consecutive MS patients undergoing AHSCT  
187 between February 2012 and February 2017 at Kings College Hospital, London. Peripheral  
188 blood stem cells were collected following standard mobilisation strategy consisting of  
189 cyclophosphamide 4g/m<sup>2</sup> over 2 days and granulocyte colony-stimulating factor for 7 days.  
190 Thirty-five (97%) patients were conditioned using standard protocol of cyclophosphamide

191 (50mg/kg for 4 days) and rATG (2.5mg/kg/day for 3 days) for in-vivo lymphodepletion;  
192 followed by stem cell infusion. One patient was conditioned with  
193 carmustine/etoposide/cytarabine/melphalan regimen along with an equivalent dose of rATG  
194 (BEAM-ATG) prior to stem cell infusion. The median CD34 stem cell dose returned~~ed~~ was 7.17  
195  $\times 10^6/\text{kg}$  (range 4.0-17.1 $\times 10^6/\text{kg}$ ).

196

197 Prior exposure to EBV was assessed by serological evidence (EBV viral capsid antigen; VCA  
198 IgG). EBV DNA load monitoring was performed on whole blood samples by standardised  
199 quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-Gene<sup>TM</sup> (Qiagen)  
200 assay of EBV BZLF1 DNA. This assay was, adapted from our published assay using  
201 LightCycler (Roche)[15] and since been validated against the recently published WHO  
202 standard, with our lab's EBV DNA quantification of 10 copies/ml considered equivalent to 10  
203 IU/ml DNA reported with the WHO reference method[16].-EBV-R was defined as rising EBV  
204 DNA load of >10 copies/millilitre (ml) detected on two consecutive tests based on our assay  
205 sensitivity. Symptomatic EBV-R was captured by peak blood EBV DNA load, presence of B  
206 symptoms (defined by presence of either unexplained weight loss, recurrent fever, night  
207 sweats); which was in-turn defined by clinical, radiological and/or histological evidence based  
208 on recent ECIL-6 guidelines[17]. In addition, significant 'clinical events' were also defined as  
209 new & persistent organ dysfunction (e.g. neurological events) temporally associated with  
210 rising EBV viraemia in MS patients. Serum protein electrophoresis was routinely tested  
211 around 3 months post HSCT; as part of our institutional practice, with immunoglobulin  
212 subclasses identified by immunofixation electrophoresis. Patient outcomes were assessed at  
213 last follow up as of April 2017.

214

215     **Statistics**

216     The database of transplants and outcomes was built in Microsoft Excel 2016 and statistical  
217     analyses were performed using IBM SPSS Statistics version 24.0. Patient characteristics are  
218     presented as medians (with inter-quartile ranges; IQR) for data with non-normal distribution.  
219     Comparisons of baseline characteristics used Mann-Whitney U test, Fisher's exact test, or  
220     Chi-squared test for trend as appropriate. Receiver Operating characteristics (ROC) curve  
221     was obtained correlating LPD and clonal gammopathy associated clinical events with rising  
222     EBV viraemia (copies/ml).

223

224     **RESULTS**

225     Baseline characteristics are presented in **Table 1**. Most MS patients (88.9%) had RRMS  
226     phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients had  
227     prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients received  
228     both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-treatment,  
229     indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. Seven MS  
230     patients were lost to long-term follow up for EBV monitoring. The median time to first EBV  
231     DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels peaked at a  
232     median of 32 days post-transplant (IQR 31-53 days). All MS patients had normal baseline  
233     lymphocyte counts (median) pre-HSCT with a median time of 46 days (range 14-404 days)  
234     to lymphocyte recovery (defined by total lymphocyte count  $>1.0 \times 10^6/\text{ml}$ ) following AHSCT  
235     (**See Figure 1**). A high proportion (86%; n=25/29) of the MS patients in active follow-up

236 recovered lymphocyte counts around D56 with a median lymphocyte count of  $1.56 \times 10^6$   
237 cells/ml); Four patients remained lymphopenic at last follow up.

238

239 All patients were stratified into following 3 groups according to peak rise/burden of EBV DNA-  
240 aemia (copies/ml): <100,000 (<100k) copies/ml, 100,001-500,00 (100k-500k) copies/ml and  
241 >500,000 (>500k) copies/ml to identify any specific thresholds for clinically significant events  
242 related to rising EBV-R (Table 1). The majority of patients (76%) with rising EBV viral load  
243 >100k copies/ml were routinely screened by computed tomographic (CT) scans to assess for  
244 evidence of LPD, in line with our institutional policy. One third (34.5%) of patients developed  
245 peak EBV viraemia of >500k copies/ml. Eight patients (27.6%) developed symptomatic EBV-  
246 R; defined as persistent fever, lymphadenopathy and/or B symptoms. Of these 8 patients,  
247 only 1 (12.5%) had a peak EBV viraemia <100kDNA copies/ml with the remaining 7 (87.5%)  
248 patients having a peak EBV viraemia of >500k copies/ml. Three patients with rising EBV  
249 viraemia >500k copies/ml had findings consistent with probable LPD on CT imaging;  
250 however, none had definitive histological diagnosis. Three MS patients had worsening  
251 neurological symptoms concurrent with rising EBV viraemia >500k copies/ml and clonal  
252 gammopathy, as described below.

253

254 Interestingly, we also observed frequent de novo monoclonal gammopathy (MG or M-protein)  
255 in 18 MS patients (62.4%) following rising EBV viraemia; the majority (n-16) of whom

256 developed IgG subtype and the remaining 2 developed IgA and IgM M-protein. Concerningly  
257 two of these patients developed clinically significant M-Protein burden; one patient with IgG  
258 Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological symptoms mimicking  
259 MS relapse, requiring plasma exchange. Another patient developed significant lumbosacral  
260 radiculopathy with rising EBV-R and high IgM lambda M-protein (IgM 48.5g/L) (**see**  
261 **supplementary case vignettes**). **Figure 2** highlights the association of neurological  
262 symptom onset following rising EBV viraemia (log copies), falling lymphocyte counts ( $\times$   
263  $10^6/\text{ml}$ ) with significant rise in M-protein (gm/lt) levels post AHSCT. A third patient developed  
264 painful lower limb paraesthesia following rising EBV viraemia  $>500\text{k}$  copies/ml, although did  
265 not have any M-protein detected. Their symptoms persisted at last follow up despite no  
266 evidence of MS related new disease activity.

267

268 Six patients were treated with anti-CD20 antibody, rituximab (375mg/m<sup>2</sup> weekly up to 4  
269 weeks), due to clinical severity of EBV reactivations and, leading to 4reduction in EBV viral  
270 load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis  
271 (**Figure 3**) confirmed EBV viraemia of  $>500\text{k}$  copies/ml correlated with high sensitivity  
272 (85.5%) and specificity (82.5%) (AUC-0.87; 95%CI-0.73-1.0; p-0.004) in predicting significant  
273 EBV related clinical events (evidence of LPD and/or neurological symptoms) that may require  
274 treatment with rituximab. The sensitivity dropped significantly on lower estimates for events  
275 below 500k copies/ml.

276

277 The median time to resolution of EBV viraemia post-rituximab was 21 days (IQR 19-124 days)  
278 in 5 patients with >500,000 ~~k~~ copies/ml (one patient was treated for late onset persistent  
279 symptomatic clonal gammopathy, despite fall in EBV levels). No significant adverse events  
280 were noted in the treated group. Nine patients had a persistent low level EBV viraemia  
281 detectable at last follow up. All patients who underwent AHSCT were alive as of April 2017.

282

## 283 **DISCUSSION:**

284 MS as an autoimmune disorder (AD) ~~has~~ is theorised to have generally similar underlying  
285 pathophysiological immune dysregulation mechanisms[18–22] relative to other chronic  
286 autoimmune conditions. Epstein-Barr virus (EBV) is increasingly implicated in the  
287 pathogenesis of MS by virtue of epidemiological and serological evidence, impaired CD8+ T  
288 cell immune responses to EBV and possible underlying genetic susceptibility for  
289 autoimmunity (with EBV encoded protein interactions), as recently described by Harley et al  
290 and others[1,23–25].

291

292 Generally, EBV-R related LPD has been reported in both allogeneic (allo-HSCT) and solid  
293 organ transplants treated with immunosuppressive therapy, often with a significant impact  
294 on organ function and overall survival[26–30]. It is observed that reduced intensity allo-HSCT  
295 for malignant haematological conditions using alemtuzumab have a relatively lower overall

296 risk of LPD compared to ATG based treatments, possibly mediated by more effective pan-B  
297 & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell repertoire with delayed  
298 EBV specific CD8+ T cell recovery[31]. Clinically significant endogenous viral infections  
299 including EBV following ATG conditioned AHSCT for severe ADs such as Crohn's disease  
300 and ~~s~~Systemic ~~s~~Sclerosis is increasingly recognised, but the development of  
301 lymphoproliferative disorders (LPD) remains rare in these ADs[13,32,33]. Nash et al[32]  
302 concerning reported 2 deaths (1 MS and 1 systemic sclerosis patient) from EBV related  
303 LPD in 56 ATG conditioned AHSCT for autoimmune diseases. Additionally, EBV associated  
304 haemophagocytosis in ATG-AHSCT for ADs have also been reported[34], with one resulting  
305 in death of the patient[35].

306

307 Our report of suspected EBV related LPDs (~10%) in MS-AHSCT group is relatively higher  
308 than published reports with allo-HSCTs (4.5-7%)[36,37] and our own centre's unpublished T-  
309 cell depleted allo-HSCT experience (6.5%); possibly a reflection of underlying  
310 immunopathological state of MS itself[38]. This is further corroborated by the fact that similar  
311 LPD risk has not been observed in other ADs, e.g. Crohn's disease, treated with ATG -  
312 AHSCT in this centre. ~~Another example from ouour~~ Another example from our ~~centre's~~ centre's experience ~~of in~~ severe aplastic  
313 anaemia ~~aa\_ (n-40), a type of AD causing severe bone marrow failure(n-40) treated & treated~~  
314 with ATG/ciclosporin, ~~n,n;~~ only 52% (n-21/40) ~~patients~~ developed EBV-R (unpublished data)  
315 and ~~-n~~None had LPD or required any treatment, ~~supporting the notion that it may not just be~~



~~a specific ATG-related problem suggesting that the problem may not be ATG specific.~~

Our study's observation of significant persistent neurological events (with no evidence of new MS disease activity) associated with clonal gammopathy, suggest a potentially new clinical syndrome, described for the first time in ATG conditioned AHSCTs in MS and, possibly induced by clonal B cell dysregulation following EBV-R. It could be hypothesised that any remaining EBV infected latent B cells, ~~which may still have survived~~surviving despite high doses of cyclophosphamide (given with mobilisation and conditioning in MS ASHCT)[8] and compounded by depletion of CD8+ T cells by ATG, may serve as potential source for EBV escape while interacting abnormally within the host immune micro-environment[39] and leading to rise in M-protein, LPD and neuro-inflammatory insults in some of the MS patients post AHSCT. Reports of lower incidence of EBV-R and LPD in another commonly used protocol for MS-AHSCT using BEAM-ATG (Ricardo Saccardi, Carregi University Hospital, Florence; personal communication) may reflect the greater myeloablative effect of BEAM chemotherapy which could further deplete the residual B cell pool and thus lower potential for EBV proliferation. It is plausible that dose of rATG is critical, given we have not seen similar reports from other centres where less rATG doses were given for MS-AHSCT (range between 5.0-6.5 mg/Kg; personal communication), but there seems to be some variability in prospective serial EBV monitoring in these patients.

336 The clinical threshold for EBV viral load as a significant risk factor for post-transplant LPD is  
337 widely debated. This study reports peak EBV viral load >500k copies/ml post AHSCT is  
338 significantly associated with probable LPD and neurological events in MS patients with high  
339 sensitivity (85.5%) and specificity (82.5%) (p=0.004) (**Fig 3, ROC curve**). Our ROC curve  
340 estimates are potentially limited by the relatively small number of events analysed but this  
341 has consistently been useful in our MS-AHSCT experience for predicting clinical events with  
342 high EBV load. Our EBV PCR assay has been validated against the recently defined standard  
343 WHO reference method (i.e. 10 copies/ml=10 IU/ml EBV DNA) [16] and thus this EBV  
344 threshold for pre-emptive treatment with Rituximab, can potentially be applied in relevant  
345 clinical context in other centres using similar validated assays. Rituximab treatment delivered  
346 good overall response in our symptomatic patients, with resolution of EBV related clinical  
347 symptoms and no subsequent viral or bacterial infections at last follow up. The role of  
348 prophylactic or pre-transplant rituximab in MS-AHSCT is also a potential area of interest in  
349 reducing risk of EBV-LPD in stem cell transplants as observed by Burns et al[36] and future  
350 randomised studies are required to investigate its potential benefit.

351

352 Our study limitations include its retrospective nature and that no suspected LPD patients had  
353 histological confirmation, mainly related to patient refusal or technical difficulties. Seven MS  
354 patients were lost to follow up for EBV monitoring following discharge, which limits the

355 findings of this study. Additionally, our numbers were too small to identify any association of  
356 EBV related clinical events with previous DMT exposure in MS patients.

357

358 In conclusion, symptomatic EBV reactivation increases risk of neurological sequelae and  
359 LPD in MS-AHSCT. Regular monitoring for rising EBV viraemia, as recommended by  
360 Snowden et al [10] and Muraro et al [11], and serum electrophoresis for M-protein should be  
361 ~~considered mandated~~ in the first 3 months post-AHSCT for MS, ~~and~~ We recommend  
362 persistent high EBV viraemia > 500k DNA copies/ml as potential trigger for consideration of  
363 pre-emptive anti-CD20 therapy and potentially reduce associated morbidity.

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370 **Acknowledgements:** To our patients and their families and carers in supporting this study.

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527 **Table 1: Baseline patient characteristics and EBV related clinical events according to peak**  
528 **EBV DNA-aemia burden.**  
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Baseline characteristics (n-36)		Patient Groups according to peak EBV DNA in copies/ml (n-29)	0 - 100,000	100,001 - 500,000	>500,000
Median age at time of AHSCT in years (range)	43.5 (36– 47)	No of patients (%)	16 (55.2)	3 (10.3)	10 (34.5)
Gender		M-Protein (n)	11	0	7
Male	19 (52.8%)				
Female	17 (47.2%)				
Disease Type (n; %)		Median EBV DNA log value at peak (IQR)			
Relapsing Remitting MS	22 (61.1%)		4.8 (3.5-4.8)	5.5 (N/A)	6.25 (6.1-6.9)
Secondary Progressive MS	10 (27.8%)				
Primary Progressive MS	4 (11.1%)				
Median number of previous DMT (range)	2 (0 – 6)	Median number of prior DMTs	2	3	2
Previous use of high efficacy DMT (n)		Symptomatic EBV (n)			
Natalizumab	22		1	0	7
Alemtuzumab	8				
Both	6				
Median EDSS (range)	6.0 (2.5 – 8.0)	LPD diagnosis (CT/Biopsy) (n)	0	0	3 by CT alone
Median follow up post AHSCT in days (range)	436 (188 – 785)	Neuro/autoimmune complications (n)	0	0	3
No. of patients with prior EBV exposure (n; %)	36 (100%)	Treated with Rituximab (n)	0	0	6
No. of patients with detectable EBV post AHSCT (n; %)	29 (80.5%)	Confirmed EBV resolution at last follow up (n)	7	2	7
No. of patients lost to long term follow up (n; %)	7 (19.5%)	Detectable EBV DNA at last follow up (n)	9	1	3
Median Time to EBV detection post AHSCT in days (IQR)	30 (23-46)	Median time for EBV resolution (IQR in days)	67 days (44-155)	47 days (N/A)	63 days (45 - 170)
Median Time to peak EBV DNA levels in days (IQR)	32 (31-53)	Median Time to peak EBV DNA levels in days (IQR)	40 days (25-85)	30 days (N/A)	39 days (32-43)

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531 **Abbreviations:**

532 AHSCT- Autologous Haematopoietic Stem cell transplant; CT- computed tomography; DMT-  
533 Disease modifying therapy; EBV- Epstein Barr virus; EDSS- Kurtzke Expanded Disability  
534 Status Scale; IQR- Interquartile range; LPD- Lymphoproliferative disease; M-Protein:  
535 Monoclonal paraprotein or gammopathy; MS- Multiple Sclerosis



## Figure Legends

### Figure 1: Trend of Lymphocyte count recovery following ATG in MS patients.

**Legend:** This figure shows trends of lymphocyte count from baseline to recovery post AHSCT for MS patients. Majority of patients had normal baseline lymphocyte counts pre-HSCT and became lymphopenic post ATG with counts recovering towards d+28 and >85% of patients recovered counts by d+56, with some overshooting from their baseline, possibly reflective of EBV related lymphoproliferation in some of these patients.

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

### Figure 2: Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT.

**Legend:** This figure demonstrates trends of EBV copies (log), paraprotein levels (g/l) and Lymphocyte levels (counts  $\times 10^6/\text{ml}$ ) in two MS patients with significant neurological symptoms following EBV reactivation. Both patients had significant EBV viraemia ( $\log > 5.2$  or  $> 500,000$  copy number) and developed significant paraproteinaemia, which was only noted after persistent unexplained neurological symptoms. The trend reversed following anti-CD20 (rituximab) therapy, with limited recovery in neurological symptoms.

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

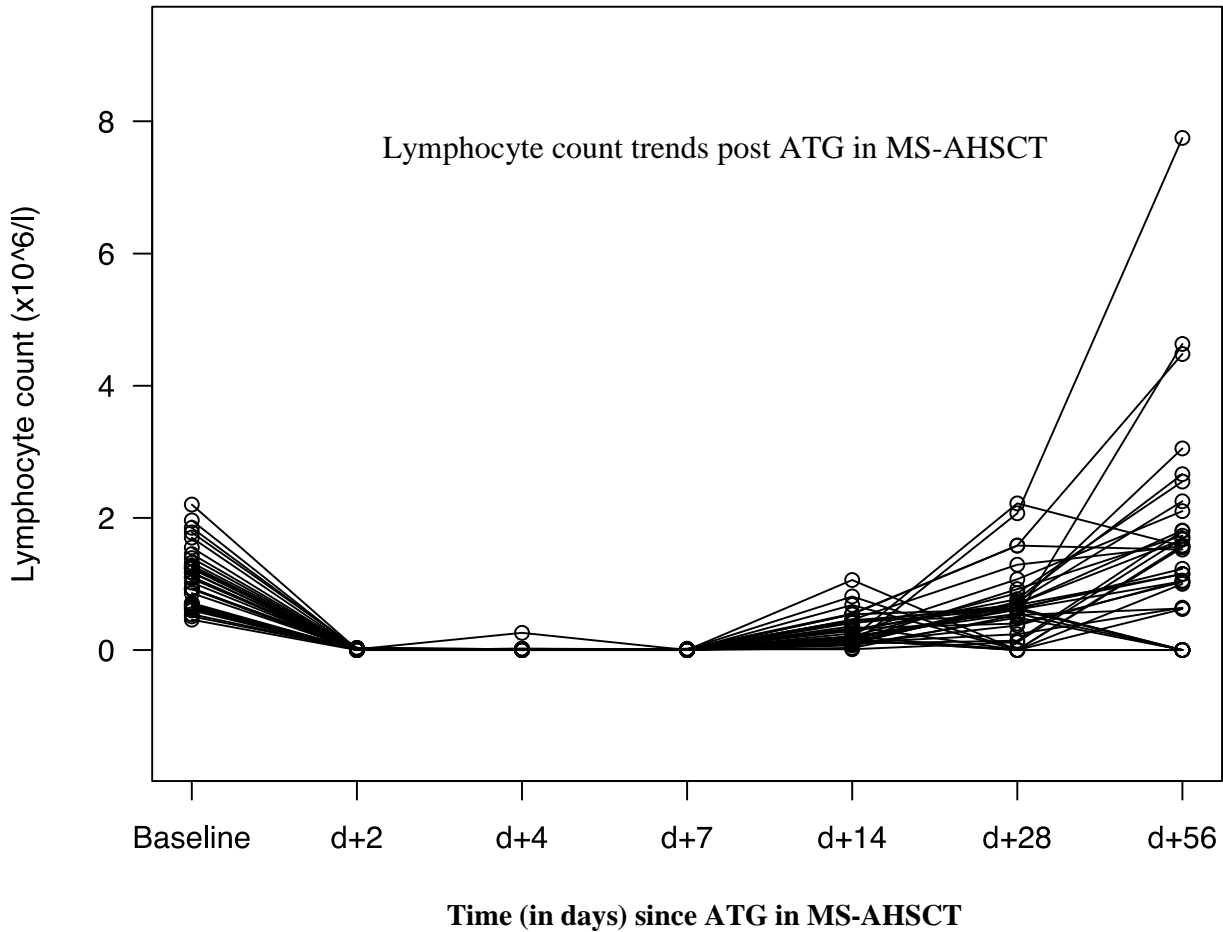
### Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.

**Legend:** ROC curve demonstrating significant correlation between high EBV levels and clinical events (LPD & neurological events) in MS-AHSCT patients, with highest sensitivity and specificity noted with peak EBV viraemia of  $> 500,000$  copies/ml ( $p = 0.0004$ ).

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

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**Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in MS patients.**

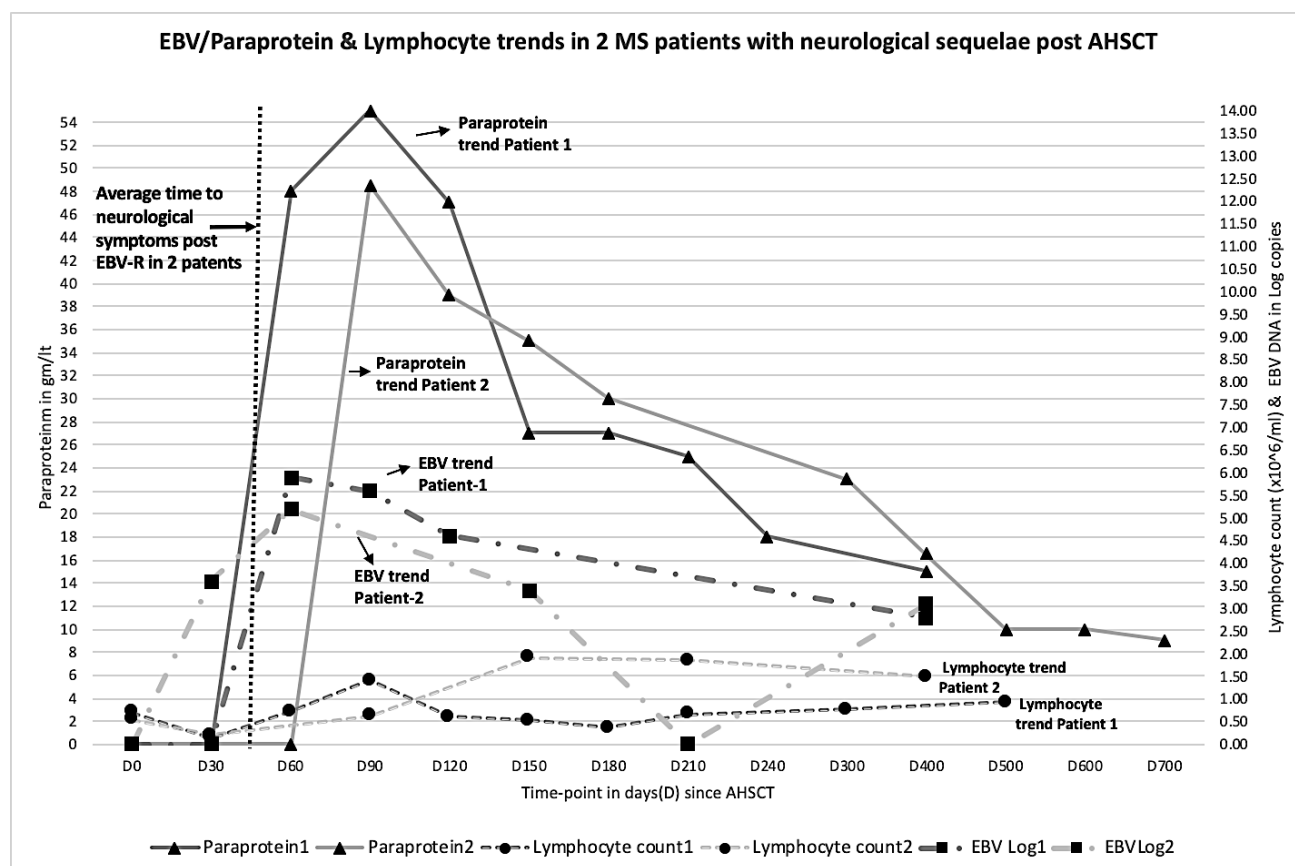


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**Abbreviations:**

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

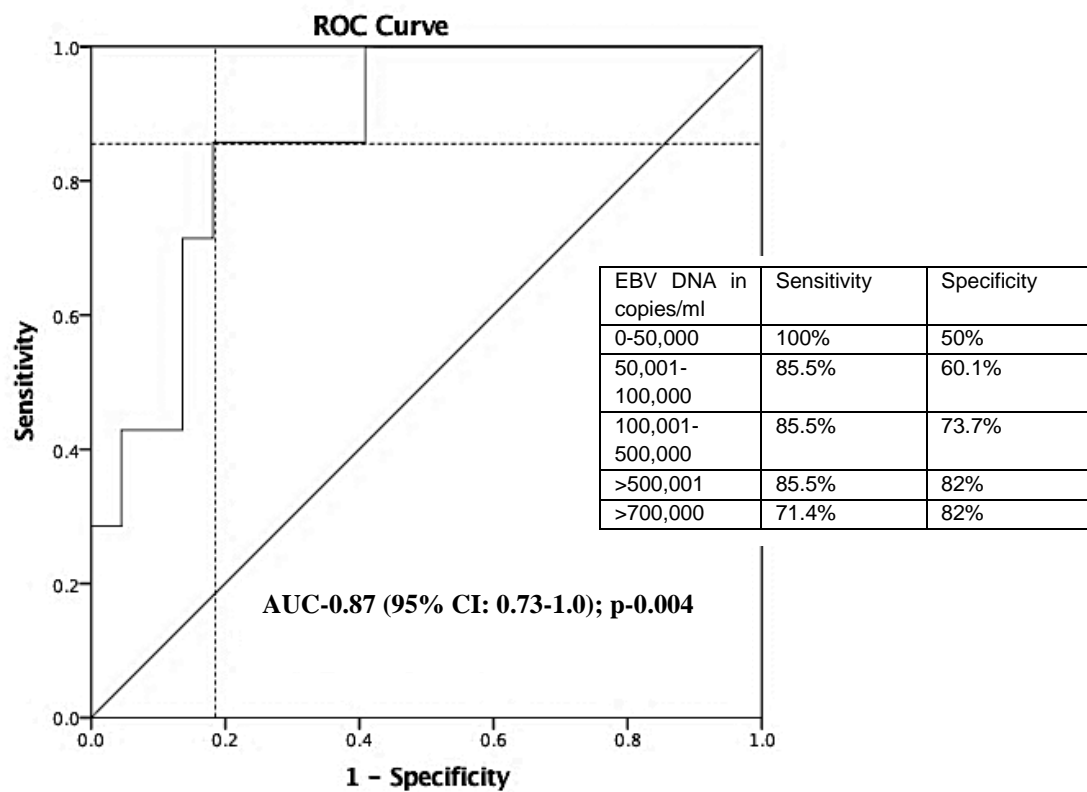
**Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT**



## Abbreviations:

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

**Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.**



### Abbreviations:

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

# **EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.**

***Running Title:*** *EBV complications in Auto-HSCT for MS*

**Varun Mehra\*, Elijah Rhone\*, Stefani Widya, Mark Zuckerman, Victoria Potter, Kavita Raj, Austin Kulasekararaj, Donal McLornan, Hugues de Lavallade, Nana Benson-Quarm, Christina Lim, Sarah Ware, Malur Sudhanva, Omar Malik, Richard Nicholas, Paolo A Muraro, Judith Marsh, Ghulam J Mufti, Eli Silber, Antonio Pagliuca and Majid A. Kazmi**

**\*These authors contributed equally to this work as 1<sup>st</sup> Authors.**

## ***Key Points:***

**EBV reactivation is common post-transplant with ATG for multiple sclerosis (MS), with significant lymphoproliferative & neurological sequelae associated with rising M-protein. Serial monitoring of EBV & M-protein is recommended post-transplant, as is pre-emptive anti-CD20 therapy with EBV DNA >500,000 copies/ml.**

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## ***Key Words:***

**Multiple Sclerosis; Autologous Hematopoietic Stem Cell Transplantation, Epstein-Barr Virus Infection; Monoclonal Gammopathy; Post-transplant Lymphoproliferative Disorder**

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78 **Conflict of interest:** The authors declare no competing financial interests as below:

- 79 1. **Varun Mehra-** no competing financial interests
- 80 2. **Elijah Rhone-** no competing financial interests
- 81 3. **Stefani Widya-** no competing financial interests
- 82 4. **Mark Zuckerman-** no competing financial interests
- 83 5. **Victoria Potter-** no competing financial interests
- 84 6. **Kavita Raj-** no competing financial interests
- 85 7. **Austin Kulasekararaj-** no competing financial interests
- 86 8. **Donal McLornan-** no competing financial interests
- 87 9. **Hugues de Lavallade-** no competing financial interests
- 88 10. **Nana Benson-Quarm-** no competing financial interests
- 89 11. **Christina Lim-** no competing financial interests
- 90 12. **Sarah Ware-** no competing financial interests
- 91 13. **Malur Sudhanva-** no competing financial interests
- 92 14. **Omar Malik-** no competing financial interests
- 93 15. **Richard Nicholas-** no competing financial interests
- 94 16. **Paolo A Muraro-** no competing financial interests
- 95 17. **Judith Marsh-** no competing financial interests
- 96 18. **Ghulam J Mufti-** no competing financial interests
- 97 19. **Eli Silber-** no competing financial interests
- 98 20. **Antonio Pagliuca-** no competing financial interests
- 99 21. **Majid A. Kazmi-** no competing financial interests

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## 111 **Abstract**

### 112 **Introduction**

113 Autologous haematopoietic stem cell transplantation (AHSCT) with anti-thymocyte globulin  
114 (ATG) conditioning as treatment of active multiple sclerosis (MS) is rapidly increasing across  
115 Europe (EBMT registry data 2017). Clinically significant Epstein Barr virus reactivation (EBV-  
116 R) following AHSCT with ATG for severe autoimmune conditions is an under-recognised  
117 complication relative to T-cell deplete transplants performed for haematological diseases.  
118 This retrospective study reports EBV-R associated significant clinical sequelae in MS patients  
119 undergoing AHSCT with rabbit ATG.

### 120 **Methods**

121 Retrospective data was analysed for 36 consecutive MS-AHSCT patients at Kings College  
122 Hospital, London. All patients routinely underwent weekly EBV DNA PCR monitoring and  
123 serum electrophoresis for monoclonal gammopathy (MG or M-protein). EBV-R with rising  
124 EBV viral load, M-protein and associated clinical sequelae were captured from clinical  
125 records.

### 126 **Results**

127 All patients had evidence of rising EBV DNA-emia, including 7 who were lost to long term  
128 follow-up, with a number of them developing high EBV viral load & associated  
129 lymphoproliferative disorder (LPD). Nearly 72% (n-18/29) developed de-novo MG, some with  
130 significant neurological consequences with high M-protein and EBV-R. Six patients required  
131 anti-CD20 therapy (rituximab) with complete resolution of EBV related symptoms. Receiver  
132 operating characteristics (ROC) estimated a peak EBV viraemia of >500,000 DNA copies/ml



133 correlated with high sensitivity (85.5%) & specificity (82.5%) (AUC-0.87; p-0.004) in  
134 predicting EBV-R related significant clinical events.

## 135 **Conclusion**

136 Symptomatic EBV reactivation increases risk of neurological sequelae and LPD in MS-  
137 AHSCT. We recommend regular monitoring for EBV and serum electrophoresis for MG in  
138 MS patients in the first 3 months post AHSCT

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## 151 INTRODUCTION:

152 Multiple sclerosis (MS) is a chronic autoimmune, inflammatory, demyelinating disease of the  
153 central nervous system[1][2], with a relapsing-remitting (RRMS) presentation in the majority  
154 of patients at diagnosis. Recovery from relapses may be complete or partial[3][4]. After a  
155 variable period of time, people with RRMS may develop a more progressive disability  
156 accumulation with or without superimposed relapses; termed secondary progressive multiple  
157 sclerosis (SPMS). A minority experience progressive disability from the onset of disease,  
158 termed primary progressive multiple sclerosis (PPMS)[4]. A number of immunomodulatory  
159 disease modifying therapies (DMTs) are currently licensed for treatment of RRMS with an  
160 aim of reducing number of relapses and accrual of disability, although with variable  
161 efficacy[5]. Since 1996, Autologous Hematopoietic Stem Cell Transplantation (AHSCT) has  
162 been a novel approach for MS management, using immunoablation followed by  
163 immunomodulation mechanisms, with evidence of significant suppression of inflammatory  
164 activity and qualitative changes in the reconstituted immune system (immune reset theory)[6–  
165 8]. AHSCT appears most effective for MS patients with evidence of inflammatory activity on  
166 MRI, younger age, a shorter disease duration, low to moderate disability levels (Expanded  
167 Disability Status Scale [EDSS] <6 or up to 6.5 if recent progression) and failure of at least 1  
168 highly active DMT (natalizumab or alemtuzumab) with no significant comorbidities[9–11].  
169 Recently reported preliminary results of randomised MIST study[12] found AHSCT to be  
170 superior to standard disease modifying therapy (DMT) for RRMS with respect to both

171 treatment failure and disability progression.

172

173 However, risk of subsequent rise in opportunistic infections following such  
174 immunosuppressive therapies remain a potential concern[13]. MS patients undergoing  
175 AHSCT have often been exposed to a number of immunomodulating DMTs; the addition of  
176 immunosuppressive rabbit anti-thymocyte globulin (rATG) to their conditioning regimen may  
177 confer a higher risk of viral reactivation in these patients. The number of AHSCTs performed  
178 for MS is rising significantly in Europe[14] and as more centres perform AHSCT for this  
179 indication, it is increasingly important to recognise the unique problems faced by these  
180 patients post AHSCT. This retrospective study reports for the first time, EBV-R associated  
181 neurological sequelae and lymphoproliferative disorder (LPD) in MS patients undergoing  
182 rATG conditioned AHSCT in our centre.

183

## 184 **METHODS**

### 185 **Patients and procedures**

186 Data was collected retrospectively on 36 consecutive MS patients undergoing AHSCT  
187 between February 2012 and February 2017 at Kings College Hospital, London. Peripheral  
188 blood stem cells were collected following standard mobilisation strategy consisting of  
189 cyclophosphamide 4g/m<sup>2</sup> over 2 days and granulocyte colony-stimulating factor for 7 days.  
190 Thirty-five (97%) patients were conditioned using standard protocol of cyclophosphamide

191 (50mg/kg for 4 days) and rATG (2.5mg/kg/day for 3 days) for in-vivo lymphodepletion  
192 followed by stem cell infusion. One patient was conditioned with  
193 carmustine/etoposide/cytarabine/melphalan regimen along with an equivalent dose of rATG  
194 (BEAM-ATG) prior to stem cell infusion. The median CD34 stem cell dose returned was 7.17  
195  $\times 10^6/\text{kg}$  (range 4.0-17.1 $\times 10^6/\text{kg}$ ).

196

197 Prior exposure to EBV was assessed by serological evidence (EBV viral capsid antigen; VCA  
198 IgG). EBV DNA load monitoring was performed on whole blood samples by standardised  
199 quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-Gene<sup>TM</sup> (Qiagen)  
200 assay of EBV BZLF1 DNA. This assay was adapted from our published assay using  
201 LightCycler (Roche)[15] and since been validated against the recently published WHO  
202 standard, with our lab's EBV DNA quantification of 10 copies/ml considered equivalent to 10  
203 IU/ml DNA reported with the WHO reference method[16]. EBV-R was defined as rising EBV  
204 DNA load of >10 copies/millilitre (ml) detected on two consecutive tests based on our assay  
205 sensitivity. Symptomatic EBV-R was captured by peak blood EBV DNA load, presence of B  
206 symptoms (defined by presence of either unexplained weight loss, recurrent fever, night  
207 sweats); which was in-turn defined by clinical, radiological and/or histological evidence based  
208 on recent ECIL-6 guidelines[17]. In addition, significant 'clinical events' were also defined as  
209 new & persistent organ dysfunction (e.g. neurological events) temporally associated with  
210 rising EBV viraemia in MS patients. Serum protein electrophoresis was routinely tested  
211 around 3 months post HSCT as part of our institutional practice, with immunoglobulin  
212 subclasses identified by immunofixation electrophoresis. Patient outcomes were assessed at  
213 last follow up as of April 2017.

214

## 215 **Statistics**

216 The database of transplants and outcomes was built in Microsoft Excel 2016 and statistical  
217 analyses were performed using IBM SPSS Statistics version 24.0. Patient characteristics are  
218 presented as medians (with inter-quartile ranges; IQR) for data with non-normal distribution.  
219 Comparisons of baseline characteristics used Mann-Whitney U test, Fisher's exact test, or  
220 Chi-squared test for trend as appropriate. Receiver Operating characteristics (ROC) curve  
221 was obtained correlating LPD and clonal gammopathy associated clinical events with rising  
222 EBV viraemia (copies/ml).

223

## 224 **RESULTS**

225 Baseline characteristics are presented in **Table 1**. Most MS patients (88.9%) had RRMS  
226 phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients had  
227 prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients received  
228 both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-treatment,  
229 indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. Seven MS  
230 patients were lost to long-term follow up for EBV monitoring. The median time to first EBV  
231 DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels peaked at a  
232 median of 32 days post-transplant (IQR 31-53 days). All MS patients had normal baseline  
233 lymphocyte counts (median) pre-HSCT with a median time of 46 days (range 14-404 days)  
234 to lymphocyte recovery (defined by total lymphocyte count  $>1.0 \times 10^6/\text{ml}$ ) following AHSCT  
235 (**See Figure 1**). A high proportion (86%; n=25/29) of the MS patients in active follow-up

236 recovered lymphocyte counts around D56 with a median lymphocyte count of  $1.56 \times 10^6$   
237 cells/ml); Four patients remained lymphopenic at last follow up.

238

239 All patients were stratified into following 3 groups according to peak rise/burden of EBV DNA-  
240 aemia (copies/ml): <100,000 (<100k) copies/ml, 100,001-500,00 (100k-500k) copies/ml and  
241 >500,000 (>500k) copies/ml to identify any specific thresholds for clinically significant events  
242 related to rising EBV-R (Table 1). The majority of patients (76%) with rising EBV viral load  
243 >100k copies/ml were routinely screened by computed tomographic (CT) scans to assess for  
244 evidence of LPD, in line with our institutional policy. One third (34.5%) of patients developed  
245 peak EBV viraemia of >500k copies/ml. Eight patients (27.6%) developed symptomatic EBV-  
246 R; defined as persistent fever, lymphadenopathy and/or B symptoms. Of these 8 patients,  
247 only 1 (12.5%) had a peak EBV viraemia <100kDNA copies/ml with the remaining 7 (87.5%)  
248 patients having a peak EBV viraemia of >500k copies/ml. Three patients with rising EBV  
249 viraemia >500k copies/ml had findings consistent with probable LPD on CT imaging;  
250 however, none had definitive histological diagnosis. Three MS patients had worsening  
251 neurological symptoms concurrent with rising EBV viraemia >500k copies/ml and clonal  
252 gammopathy, as described below.

253

254 Interestingly, we also observed frequent de novo monoclonal gammopathy (MG or M-protein)  
255 in 18 MS patients (62.4%) following rising EBV viraemia; the majority (n=16) of whom

256 developed IgG subtype and the remaining 2 developed IgA and IgM M-protein. Concerningly  
257 two of these patients developed clinically significant M-Protein burden; one patient with IgG  
258 Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological symptoms mimicking  
259 MS relapse, requiring plasma exchange. Another patient developed significant lumbosacral  
260 radiculopathy with rising EBV-R and high IgM lambda M-protein (IgM 48.5g/L) (**see**  
261 **supplementary case vignettes**). **Figure 2** highlights the association of neurological  
262 symptom onset following rising EBV viraemia (log copies), falling lymphocyte counts ( $\times$   
263  $10^6/\text{ml}$ ) with significant rise in M-protein (gm/l) levels post AHSCT. A third patient developed  
264 painful lower limb paraesthesia following rising EBV viraemia  $>500\text{k}$  copies/ml, although did  
265 not have any M-protein detected. Their symptoms persisted at last follow up despite no  
266 evidence of MS related new disease activity.

267

268 Six patients were treated with anti-CD20 antibody, rituximab (375mg/m<sup>2</sup> weekly up to 4  
269 weeks), due to clinical severity of EBV reactivations and leading to reduction in EBV viral  
270 load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis  
271 (**Figure 3**) confirmed EBV viraemia of  $>500\text{k}$  copies/ml correlated with high sensitivity  
272 (85.5%) and specificity (82.5%) (AUC-0.87; 95%CI-0.73-1.0; p-0.004) in predicting significant  
273 EBV related clinical events (evidence of LPD and/or neurological symptoms) that may require  
274 treatment with rituximab. The sensitivity dropped significantly on lower estimates for events  
275 below 500k copies/ml.

276

277 The median time to resolution of EBV viraemia post-rituximab was 21 days (IQR 19-124 days)  
278 in 5 patients with >500k copies/ml (one patient was treated for late onset persistent  
279 symptomatic clonal gammopathy, despite fall in EBV levels). No significant adverse events  
280 were noted in the treated group. Nine patients had a persistent low level EBV viraemia  
281 detectable at last follow up. All patients who underwent AHSCT were alive as of April 2017.

282

## 283 **DISCUSSION:**

284 MS as an autoimmune disorder (AD) is theorised to have generally similar underlying  
285 pathophysiological immune dysregulation mechanisms[18–22] relative to other chronic  
286 autoimmune conditions. Epstein-Barr virus (EBV) is increasingly implicated in the  
287 pathogenesis of MS by virtue of epidemiological and serological evidence, impaired CD8+ T  
288 cell immune responses to EBV and possible underlying genetic susceptibility for  
289 autoimmunity (with EBV encoded protein interactions), as recently described by Harley et al  
290 and others[1,23–25].

291

292 Generally, EBV-R related LPD has been reported in both allogeneic (allo-HSCT) and solid  
293 organ transplants treated with immunosuppressive therapy, often with a significant impact  
294 on organ function and overall survival[26–30]. It is observed that reduced intensity allo-HSCT  
295 for malignant haematological conditions using alemtuzumab have a relatively lower overall



296 risk of LPD compared to ATG based treatments, possibly mediated by more effective pan-B  
297 & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell repertoire with delayed  
298 EBV specific CD8+ T cell recovery[31]. Clinically significant endogenous viral infections  
299 including EBV following ATG conditioned AHSCT for severe ADs such as Crohn's disease  
300 and systemic sclerosis is increasingly recognised, but the development of lymphoproliferative  
301 disorders (LPD) remains rare in these ADs[13,32,33]. Nash et al[32] concerningly reported  
302 2 deaths (1 MS and 1 systemic sclerosis patient) from EBV related LPD in 56 ATG  
303 conditioned AHSCT for autoimmune diseases. Additionally, EBV associated  
304 haemophagocytosis in ATG-AHSCT for ADs have also been reported[34], with one resulting  
305 in death of the patient[35].

306

307 Our report of suspected EBV related LPDs (~10%) in MS-AHSCT group is relatively higher  
308 than published reports with allo-HSCTs (4.5-7%)[36,37] and our own centre's unpublished T-  
309 cell depleted allo-HSCT experience (6.5%); possibly a reflection of underlying  
310 immunopathological state of MS itself[38]. This is further corroborated by the fact that similar  
311 LPD risk has not been observed in other ADs, e.g. Crohn's disease, treated with ATG -  
312 AHSCT in this centre. Another example from our centre's experience of severe aplastic  
313 anaemia (n=40) treated with ATG/ciclosporin, only 52% (n=21/40) developed EBV-R  
314 (unpublished data) and none had LPD or required any treatment, suggesting that the problem  
315 may not be ATG specific.

316

317 Our study's observation of significant persistent neurological events (with no evidence of new  
318 MS disease activity) associated with clonal gammopathy suggest a potentially new clinical  
319 syndrome, described for the first time in ATG conditioned AHSCTs in MS and possibly  
320 induced by clonal B cell dysregulation following EBV-R. It could be hypothesised that any  
321 remaining EBV infected latent B cells, surviving despite high doses of cyclophosphamide  
322 (given with mobilisation and conditioning in MS ASHCT)[8] and compounded by depletion of  
323 CD8+ T cells by ATG, may serve as potential source for EBV escape while interacting  
324 abnormally within the host immune micro-environment[39] and leading to rise in M-protein,  
325 LPD and neuro-inflammatory insults in some of the MS patients post AHSCT. Reports of  
326 lower incidence of EBV-R and LPD in another commonly used protocol for MS-AHSCT using  
327 BEAM-ATG (Ricardo Saccardi, Carregi University Hospital, Florence; personal  
328 communication) may reflect the greater myeloablative effect of BEAM chemotherapy which  
329 could further deplete the residual B cell pool and thus lower potential for EBV proliferation. It  
330 is plausible that dose of rATG is critical, given we have not seen similar reports from other  
331 centres where less rATG doses were given for MS-AHSCT (range between 5.0-6.5 mg/Kg;  
332 personal communication) but there seems to be some variability in prospective serial EBV  
333 monitoring in these patients.

334

335 The clinical threshold for EBV viral load as a significant risk factor for post-transplant LPD is  
336 widely debated. This study reports peak EBV viral load >500k copies/ml post AHSCT is  
337 significantly associated with probable LPD and neurological events in MS patients with high  
338 sensitivity (85.5%) and specificity (82.5%) (p=0.004) **(Fig 3, ROC curve)**. Our ROC curve  
339 estimates are potentially limited by the relatively small number of events analysed but this  
340 has consistently been useful in our MS-AHSCT experience for predicting clinical events with  
341 high EBV load. Our EBV PCR assay has been validated against the recently defined standard  
342 WHO reference method (i.e. 10 copies/ml=10 IU/ml EBV DNA) [16] and thus this EBV  
343 threshold for pre-emptive treatment with Rituximab, can potentially be applied in relevant  
344 clinical context in other centres using similar validated essays. Rituximab treatment delivered  
345 good overall response in our symptomatic patients, with resolution of EBV related clinical  
346 symptoms and no subsequent viral or bacterial infections at last follow up. The role of  
347 prophylactic or pre-transplant rituximab in MS-AHSCT is also a potential area of interest in  
348 reducing risk of EBV-LPD in stem cell transplants as observed by Burns et al[36] and future  
349 randomised studies are required to investigate its potential benefit.

350

351 Our study limitations include its retrospective nature and that no suspected LPD patients had  
352 histological confirmation, mainly related to patient refusal or technical difficulties. Seven MS  
353 patients were lost to follow up for EBV monitoring following discharge, which limits the

354 findings of this study. Additionally, our numbers were too small to identify any association of  
355 EBV related clinical events with previous DMT exposure in MS patients.

356

357 In conclusion, symptomatic EBV reactivation increases risk of neurological sequelae and  
358 LPD in MS-AHSCT. Regular monitoring for rising EBV viraemia, as recommended by  
359 Snowden et al [10] and Muraro et al [11], and serum electrophoresis for M-protein should be  
360 considered in the first 3 months post-AHSCT for MS. We recommend persistent high EBV  
361 viraemia > 500k DNA copies/ml as potential trigger for consideration of pre-emptive anti-  
362 CD20 therapy and potentially reduce associated morbidity.

363

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368

369 **Acknowledgements:** To our patients and their families and carers in supporting this study.

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526 **Table 1: Baseline patient characteristics and EBV related clinical events according to peak**  
527 **EBV DNA-aemia burden.**  
528

Baseline characteristics (n-36)		Patient Groups according to peak EBV DNA in copies/ml (n-29)	0 - 100,000	100,001 - 500,000	>500,000
Median age at time of AHSCT in years (range)	43.5 (36– 47)	No of patients (%)	16 (55.2)	3 (10.3)	10 (34.5)
Gender		M-Protein (n)	11	0	7
Male	19 (52.8%)				
Female	17 (47.2%)				
Disease Type (n; %)		Median EBV DNA log value at peak (IQR)			
Relapsing Remitting MS	22 (61.1%)		4.8 (3.5-4.8)	5.5 (N/A)	6.25 (6.1-6.9)
Secondary Progressive MS	10 (27.8%)				
Primary Progressive MS	4 (11.1%)				
Median number of previous DMT (range)	2 (0 – 6)	Median number of prior DMTs	2	3	2
Previous use of high efficacy DMT (n)		Symptomatic EBV (n)			
Natalizumab	22		1	0	7
Alemtuzumab	8				
Both	6				
Median EDSS (range)	6.0 (2.5 – 8.0)	LPD diagnosis (CT/Biopsy) (n)	0	0	3 by CT alone
Median follow up post AHSCT in days (range)	436 (188 – 785)	Neuro/autoimmune complications (n)	0	0	3
No. of patients with prior EBV exposure (n; %)	36 (100%)	Treated with Rituximab (n)	0	0	6
No. of patients with detectable EBV post AHSCT (n; %)	29 (80.5%)	Confirmed EBV resolution at last follow up (n)	7	2	7
No. of patients lost to long term follow up (n; %)	7 (19.5%)	Detectable EBV DNA at last follow up (n)	9	1	3
Median Time to EBV detection post AHSCT in days (IQR)	30 (23-46)	Median time for EBV resolution (IQR in days)	67 days (44-155)	47 days (N/A)	63 days (45 - 170)
Median Time to peak EBV DNA levels in days (IQR)	32 (31-53)	Median Time to peak EBV DNA levels in days (IQR)	40 days (25-85)	30 days (N/A)	39 days (32-43)

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530 **Abbreviations:**

531 AHSCT- Autologous Haematopoietic Stem cell transplant; CT- computed tomography; DMT-  
532 Disease modifying therapy; EBV- Epstein Barr virus; EDSS- Kurtzke Expanded Disability  
533 Status Scale; IQR- Interquartile range; LPD- Lymphoproliferative disease; M-Protein:  
534 Monoclonal paraprotein or gammopathy; MS- Multiple Sclerosis

## Figure Legends

### Figure 1: Trend of Lymphocyte count recovery following ATG in MS patients.

**Legend:** This figure shows trends of lymphocyte count from baseline to recovery post AHSCT for MS patients. Majority of patients had normal baseline lymphocyte counts pre-HSCT and became lymphopenic post ATG with counts recovering towards d+28 and >85% of patients recovered counts by d+56, with some overshooting from their baseline, possibly reflective of EBV related lymphoproliferation in some of these patients.

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

### Figure 2: Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT.

**Legend:** This figure demonstrates trends of EBV copies (log), paraprotein levels (g/l) and Lymphocyte levels (counts  $\times 10^6/\text{ml}$ ) in two MS patients with significant neurological symptoms following EBV reactivation. Both patients had significant EBV viraemia (log>5.2 or >500,000 copy number) and developed significant paraproteinaemia, which was only noted after persistent unexplained neurological symptoms. The trend reversed following anti-CD20 (rituximab) therapy, with limited recovery in neurological symptoms.

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

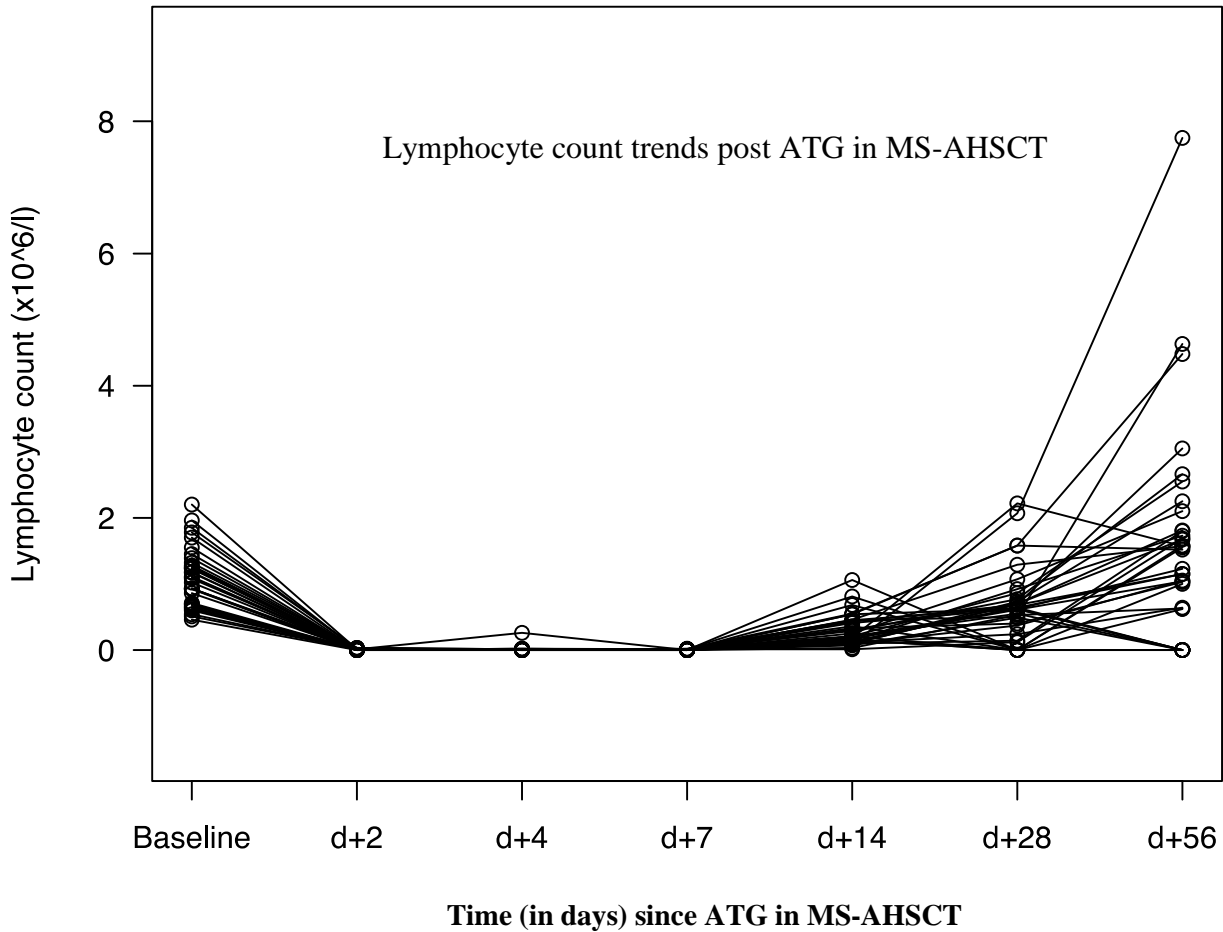
### Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.

**Legend:** ROC curve demonstrating significant correlation between high EBV levels and clinical events (LPD & neurological events) in MS-AHSCT patients, with highest sensitivity and specificity noted with peak EBV viraemia of >500,000 copies/ml (p=0.0004).

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

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**Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in MS patients.**

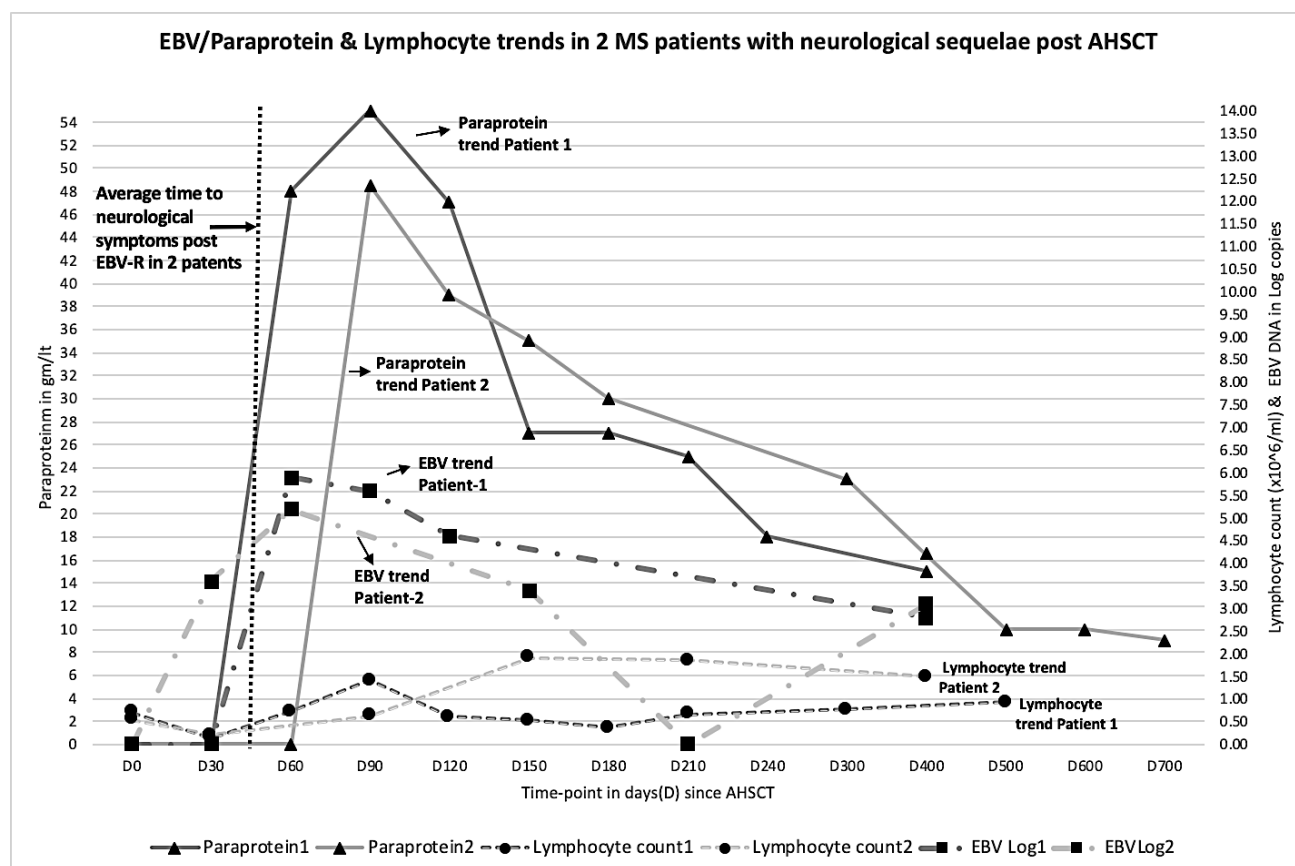


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**Abbreviations:**

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

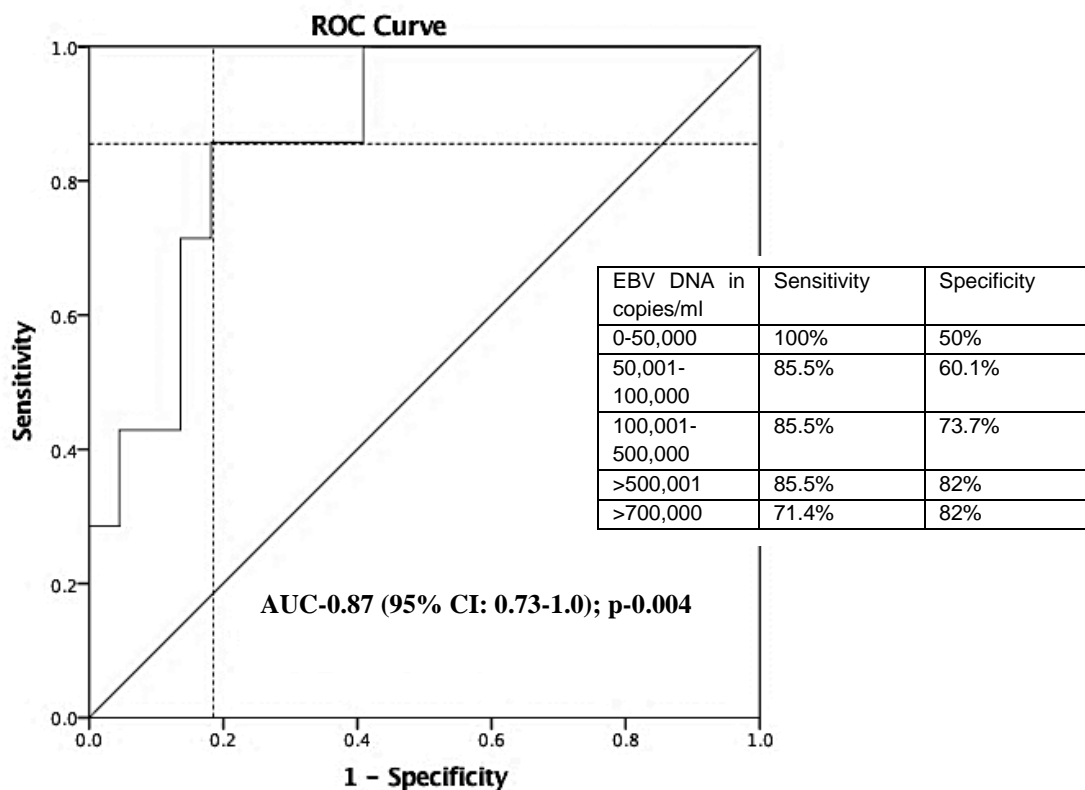
**Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT**



## Abbreviations:

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

**Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.**



#### **Abbreviations:**

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

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